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ERRATA

On Page 265, Vol 79, No 3, November 1943, References (1) and (7),
"MacIntosh" should be changed to "Macintosh"

In preparing Volume Contents and Index for Vol 80, No 4 (April 1944)
the article beginning on page 383 of that number was inadvertently omitted
To complete the same, clip the following entries and paste on pages 409, 412
and v of that number.

CORRECTION

On page 317, Vol 79, No 4, December 1943,
lines 2 and 3, the figures should read 0 8 and
0 85 instead of 0 3 and 0.35 respectively

VASCULAR FRAGILITY AND PERMEABILITY AS INFLUENCED BY VARIOUS AGENTS

I A DESCRIPTION OF AN EXPERIMENTAL METHOD AND OF THE EFFECTS OF VARIOUS SUBSTANCES RELATED TO VITAMIN P[1]

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Szent Gyorgy's report (1) of a new vitamin, which he designated as the permeability vitamin (vitamin P) followed the clinical observation that a concentrate of paprika controlled hemorrhages in patients who did not respond to vitamin C   He then found that an extract of lemon had the same effect as paprika (2, 3)   This material, which he called ' citrin" was thought to be a mixture of hesperidin and eriodictyol glycoside   Altogether, Szent Gyorgyi and his associates treated 3 cases of vascular purpura, 4 cases of thrombocytopenic purpura, 7 cases of infectious diseases, 2 cases of diabetes mellitus and 1 case of myxedema

Scarborough and Steward (4) and Scarborough (5) treated a number of cases of patients who showed an increase in capillary fragility, based upon dietary deficiencies, and who showed no improvement on addition of vitamins A, B₁, C or D to the diet   They used oranges, a crude hesperidin from valencia oranges, a pyridine extract of the crude hesperidin, and lemon juice, and an injectable extract of lemons made according to Szent Gyorgyi's method for "citrin"   All were effective in diminishing cutaneous vascular fragility   Vascular fragility was determined either by the positive pressure method wherein a blood pressure cuff is applied to an extremity and inflated to a pressure between venous and arterial blood pressure, or by applying suction to several restricted areas of skin, for a period of one minute

Rapaport and Klein (6) reduced cutaneous vascular fragility in allergic children with ' calcium eriodictate" and Goldfarb (7) relieved cases of psoriasis with ' citrin crystals" and with an aqueous extract of whole lemon, called "citrin lemonade"   Unfortunately, the true chemical nature of "calcium eriodictate" and of "citrin crystals" is not established

Our investigations on vitamin P began with a water soluble extract of lemon peel, designated as A 52   This we found to have definite pharmacological properties, in that it caused an acetylcholine like depression of blood pressure in cats and rabbits, and altered rhythm of the isolated frog heart (8)   Armentano (9) reported a lowering of blood pressure with citrin   Similar blood pressure effects

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have been observed here with crude orange hesperidin but not with purified hesperidin.[2]

In view of the clinical reports on alteration of vascular permeability or fragility by various citrus products, one of us[3] investigated three methods for the experimental study of this problem. First, because of the supposed relation between the adrenals and permeability, A-52 was tested for a possible effect on general tissue permeability as follows: young rats were adrenalectomized and given a daily subminimal dose of desoxycorticosterone[4] by subcutaneous injection in sesame oil; half of these rats received daily subcutaneous doses of lemon product A-52 and the other half received daily injections of equal amounts of saline solution. Both length of life after adrenalectomy (survival time) and the rate of growth were observed. Because of inconstant results, this method was discarded although our impression was that growth was more rapid in the group receiving A-52.

The second method, which also gave suggestively positive results consisted in applying suction for one minute with a 4 mm. glass tube to the conjunctiva of the eyelid of a rabbit and counting resulting petechiae. An injection of saline was made under the conjunctiva of one lid and of saline containing A-52 under the opposite eye lid. These results were also irregular and the method was discarded.

A third method, to be described below, based upon the production of lung hemorrhage when mice are subjected to a sudden decrease in atmospheric pressure to 70 mm, yielded much more definite results and hence has become a standard method in this laboratory for testing alterations of vascular fragility by various lemon and other products. Hemorrhage is much reduced by oral administration of potent materials having vitamin P properties.

SOURCE OF MATERIALS. The three materials investigated were obtained from the Research Department of the California Fruit Growers Exchange. Product A-52 was obtained by extracting ground lemons with hot ethyl alcohol. The alcohol was evaporated, the solution filtered, concentrated and extracted with isopropanol. The isopropanol solution was evaporated to small volume and two volumes of petroleum ether were added. A precipitate formed which was dried, dissolved in hot absolute isopropanol and reprecipitated by chilling. This was then dissolved in water.

Crude hesperidin (168-9) was extracted from orange peel with sodium hydroxide, the alkaline liquid was screened and neutralized with hydrochloric acid. A precipitate formed and this was separated by centrifugation and was dried.

The sample of purified hesperidin used in most tests was labelled A-30, m.p. 255-256.

METHOD. White mice of approximately 20 grams weight were used. Sex did not seem to influence the results, although any one experiment was carried out on either all males

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[2] Data to be reported later.

[3] A. J. Lesser.

[4] Percorten, kindly supplied by Ciba Pharmaceutical Products, Inc.

or all females The animals were taken directly from the stock cage for testing Animals subjected to the test later than one hour after administration of the test substance were allowed access to food and water during the interval The composition of this stock diet was as follows 40 parts yellow corn meal 28 parts ground whole wheat 18 parts dry skim milk 4 parts alfalfa meal, 6 parts linseed meal, 2 parts cod liver oil 1 part dried brewers' yeast, 1 part fish meal 0 5 part sodium chloride 0 5 part calcium carbonate by weight

Product A 52 was administered both intragastrically by means of a large gauge blunt needle passed down the esophagus and intraperitoneally in a dose of one or two milligrams per gram body weight The volume was kept under 1 cc Tests were made at one, two, four and twenty four hour intervals Since crude hesperidin and purified hesperidin are not water soluble they were suspended in water and given intragastrically in doses of one or two milligrams per gram weight of mouse Control mice received water in equal volume

Fig 1 Photograph of lung of control mouse (left) and lung of mouse treated with crude hesperidin 168–9 (right) Control lung shows 100 per cent hemorrhage and treated lung no hemorrhage

The test was performed by placing equal numbers of control and test animals in a small pyrex dome topped dessicator jar, connected by means of a stop cock and pressure tubing to a five gallon bottle The large bottle had been previously connected to the vacuum line and evacuated to 45 mm of mercury pressure Then the stop cock to the dessicator was quickly opened The pressure changed to 70 mm The mice died within 15 to 60 seconds Sixty seconds after opening the stop cock it was again closed the dessicator lid was removed 1 minute later and the mice taken out for examination The abdomen and chest were quickly opened and observations made of the extent of pulmonary hemorrhage

Control mice subjected to this treatment exhibited hemorrhages into the lungs which when seen from the lung surface varied in appearance from petechiae to diffuse extravasation and were rated from 0 to ++++ At first drawings of the lungs and hemorrhagic areas were made but later it became possible to classify the degrees of hemorrhage into 4 grades by direct observation Figure 1 illustrates the appearance of a control lung and of the lung of an animal given crude hesperidin

The lungs of mice receiving protective materials were either clear of apparent hemorrhage or exhibited hemorrhage of much less degree than the control animals

RESULTS   Table 1 summarizes the data The figures representing degree of hemorrhage were obtained by averaging the group and multiplying by 25 Complete hemorrhage then had a value of 100, complete protection, a value of 0

TABLE 1

Summary of degrees of pulmonary hemorrhage as influenced by various forms of "vitamin P"

SUBSTANCE AND MANNER OF ADMINIS TRATION	AVERAGE DEGREE OF PULMONARY HEMORRHAGE								CON TROLS* FOR A 52
	1 hour		2 hours		4 hours		24 hours		
A 52, 1 mgm /gm intragastri cally	17 (6)		25 (3)		(3 hrs) 100 (4)		100 (4)		75 (7)
		Con		Con		Con		Con	
Crude hesperidin 168–9, 2 mgm / gm intragas trically	17 (9)	85 (10)			70 (5)	79 (6)	28 (7)	41 (7)	
Purified hesperi din A 30, 1 mgm /gm in tragastrically	92 (3)	50 (3)							
Purified hesperid in A 30, 2 mgm /gm intragas trically	94 (9)	67 (10)	75 (3)	46 (3)	50 (7)	61 (7)	35 (6)	43 (7)	

* Controls for A 52 were not tested at the same time as the treated animals With the hesperidin preparations, control and treated animals were tested simultaneously Numbers in parenthesis are the actual number of mice for each test

TABLE 2

Influence of crude hesperidin on pulmonary hemorrhage in individual mice

	2 MGM /GM	CONTROLS
1 hour	+ 0 0 0 + + + + +	+++* ++++ ++ +++ +++ +++ ++++ ++++ ++++ ++++
4 hours	++++ + ++ ++++ +++	+ ++++ ++++ ++ ++++ ++++
24 hours	+++ + ++ ± ± ± ±	++ + ++ ++++ ± ±

* Plus signs indicate degree of hemorrhage, ± indicates very slight hemorrhage In table 1, each + is given a value of 25 and ± a value of 12 5

Table 2 is inserted to illustrate the individual variability of hemorrhagic responses of mice used in testing crude hesperidin (168–9)

It is apparent that substance A-52 was rapidly effective but that its effect was lost within 3 hours   Crude hesperidin was also rapidly effective but variable
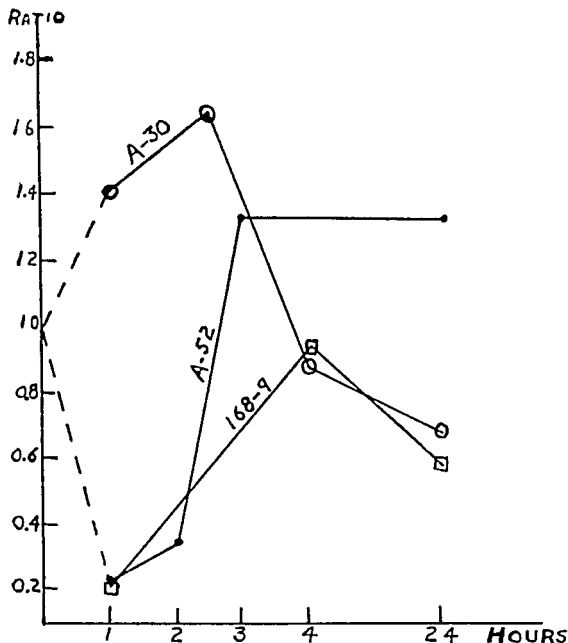
FIG 2 Graphs showing ratios of degree of lung hemorrhage of treated mice to degree of lung hemorrhage of control mice at intervals after treatment   Calculated from table 1 A 52 ●——●, A 30 ○——○, 168-9, □——□

in the duration of effect, in that there was an early protective effect similar to that of A 52, which was lost at 4 hours   At 24 hours, the calculated figures suggest a slight protective effect again   Mice receiving purified hesperidin exhibited a greater degree of hemorrhage at 1 and 2 hours, and showed questionable or no protection at 4 and 24 hours

For the benefit of those who may wish to repeat or extend our work, it must be stated that there have been occasional control mice which failed to exhibit pulmonary hemorrhage. We have made some progress in explaining this peculiarity on the basis of presence or absence of food in the stomach and possibly on the basis of flow of bile. This will be discussed in detail in a subsequent report. Occasionally, axillary or other hemorrhage occurred; in such mice, pulmonary hemorrhage was slight, but these animals were discarded from the experiment.

DISCUSSION. The above results are of interest especially since certain clinical evidence points to the control of clinical hemorrhage with "vitamin P" even in cases which do not show an improvement in fragility of skin vessels. Our own experiments on fragility of skin vessels in experimental animals (10) have not progressed far enough to make comparisons with the lung method. It is reasonable to predict that the lung hemorrhage technique would give data more nearly applicable to internal hemorrhages in patients than would the skin tests.

Another point of interest is that these unknown substances were effective in animals raised on a supposedly complete diet. This diet has been the standard stock food for mice and rats in the Department for a number of years, and has been shown to be adequate for satisfactory growth and fertility. In order to determine whether this stock diet contains "vitamin P" a colony of mice is now being given the Sherman-La Mer diet relatively free of flavones, and the pulmonary vascular resistance of these is to be compared with animals raised on the stock diet. In view of the responses (blood pressure, heart, vascular fragility) to these substances of animals fed a supposedly complete diet, the present experiments cannot be used to support the concept that the active substance or substances should be classed with the vitamins. Results of other types of experiments, to be reported later, however, more satisfactorily support the vitamin nature of these citrus products.

Finally, it is of interest that the effects with A-52 and with crude hesperidin were quickly apparent (1 hour or less). Hiramatsu (11) reported protective effects in sensitized guinea pigs 30 minutes after injections of vitamin P.

SUMMARY

A method has been developed which can be used to show the protective action of certain citrus peel fractions ("vitamin P") in controlling vascular fragility. When air is suddenly evacuated from a jar in which mice are placed, hemorrhage occurs into the lungs of these mice. Crude hesperidin obtained from oranges, and a water soluble extract of lemon peel afforded protection to mice against this hemorrhage for a period of 2 to 4 hours after administration. Pure hesperidin had little or no protective effect, but during the first 2 hours following its administration, a greater degree of hemorrhage was apparent.

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STUDIES ON PHYSOSTIGMINE AND RELATED SUBSTANCES[1]

I. QUANTITATIVE RELATION BETWEEN DOSAGE OF PHYSO-STIGMINE AND INHIBITION OF CHOLINESTERASE ACTIVITY IN THE BLOOD SERUM OF DOGS

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Since Loewi's discovery of the inhibitory action of physostigmine on cholinesterase activity (1) the term "eserinized" has entered into general usage. The word is usually used to denote experimental conditions involving inhibition of cholinesterase activity. In reality, very few investigators have studied the actual degree of cholinesterase activity reached in their experiments. For example, in the experiments of Feldberg and Krayer (2) and Krayer and Verney (3), nothing was known of the intensity and duration of the specific action of physostigmine given for the purpose of studying changes of acetylcholine concentration in the coronary blood of dogs upon vagal stimulation. Ammon and Voss (4) estimated the change in cholinesterase activity of serum and whole blood in a single dog of 17 kgm. which had received 5 mgm. of physostigmine sulfate [a dose of the order used by Krayer and Verney (3)], and 20 minutes after the injection found the cholinesterase activity in the serum decreased. Jones and Tod (5) gave 1.3 mgm. of physostigmine sulfate subcutaneously to 12 patients, took a sample of blood within 10 to 35 minutes after the injection, and found a decrease in cholinesterase activity of the serum upon comparing it with normal serum activity. Manning, Lang, and Hall (6) were the first to follow the degree of cholinesterase inhibition of serum in dogs after the injection of single doses (0.05 to 0.1 mgm. per kgm. intravenously; 0.25 mgm. per kgm. subcutaneously) and during the continuous intravenous administration of 1.66 micrograms of physostigmine sulfate per kgm. per minute. The effect of continuous intravenous infusion upon cholinesterase activity in cats was examined by Rentz (7), who compared the action of 0.5 to 60 micrograms of physostigmine salicylate per kgm. of body weight per minute with that of methylene blue and trypaflavin.

We have found no record of sufficiently comprehensive work giving the detailed time course of the inhibition of serum cholinesterase activity after single doses of physostigmine, nor any comprehensive work relating the rate of continuous infusion to the level of inhibition when the animal organism, with regard to the physostigmine concentration, is in a state of dynamic equilibrium such that the serum cholinesterase activity remains at a constant level. The present experiments are a study of the quantitative relation between the amount

of physostigmine administered and the resulting decrease in cholinesterase activity of the blood serum of dogs after single injections and during continuous infusions of physostigmine salicylate

METHODS   The experiments were conducted in adult dogs weighing 18 to 27 5 kgm Animals of this size were selected because samples totalling as much as 150 cc of blood had to be taken during the course of the experiment   Chloralose anesthesia was abandoned after a short trial because an excessive amount of saline was necessary to dissolve the repeated doses needed during a continuous infusion experiment   Satisfactory anesthesia was induced by 0 04 grams of nembutal per kgm of body weight intraperitoneally and maintained by 0 1 to 0 2 gram doses in 20 to 50 cc of 0 9% sodium chloride solution infused into the left femoral vein as needed   In control experiments we found no significant change in serum cholinesterase activity during many hours of nembutal anesthesia   The animals were placed on a warm table   rectal temperature was recorded, and care was taken to maintain body temperature as nearly constant as possible throughout the experiment Respiration was spontaneous except for short periods in a few experiments   Respiration rate and heart rate were counted frequently   Except in dogs that were to survive, a cannula was placed in the left femoral artery and connected to a mercury manometer   blood pressure records were taken at hourly intervals   The hematocrit of every blood sample was measured and enough 0 9% sodium chloride solution was infused to prevent hemoconcentration

Serum cholinesterase was determined by the manometric method of Friend and Krayer (8)   The reaction mixture contained 22 2% serum and 2 7% acetylcholine bromide[2] buffered to pH 7 4 at 38°C   The absolute units represent millimoles of carbon dioxide produced per liter of reaction mixture in 20 minutes   (Our units $\times$ 13 5 = millimoles of acetylcholine hydrolyzed per liter of serum per hour at pH 7 4 and 38°C)   Enzymatic hydrolysis proceeds at a maximum and constant velocity during this period   The enzyme activity is therefore the limiting factor throughout

Straus and Goldstein (9) have studied the effect of dilution upon the percentage activity of an enzyme in an enzyme inhibitor system   and according to their findings the cholinesterase physostigmine system falls into a zone of behavior in which dilution is a critical procedure and reduces markedly the degree of inhibition, thus actual values as observed in the reaction mixture of the gas analyzer require correction to true serum cholinesterase activity values   The significance of this correction and of certain others will be discussed fully below   together with their limitations and probable inaccuracies   Unless otherwise stated   all activity values mentioned in the text of this paper are corrected

Blood samples of 10 cc each were taken under liquid paraffin from the exposed left jugular vein   The blood was defibrinated, centrifuged and the serum stored at 6°C   from 1 to 24 hours until the determinations were made   No measurable change in physostigmine concentration (decrease in inhibition of cholinesterase activity) occurs during this storage period at a low temperature

Control blood samples were obtained from a foreleg vein one to three days before the experiments and again from the jugular vein after anesthesia but before physostigmine was given   These as a rule were within 10% of each other and were often much closer Their average was considered the   normal' cholinesterase activity, and variations from this were expressed in percent of the normal   A single 200 cc bleeding under anesthesia performed at the beginning of a control period (see figure 1) had no demonstrable effect on cholinesterase activity in 5 hours   This volume of blood is larger than the sum of all the 10 cc samples taken in any other experiment

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[2] The acetylcholine bromide used in these experiments was generously provided by Hoffmann La Roche Inc , Nutley N J

Physostigmine salicylate[3] dissolved in 0.9% sodium chloride solution was used in all experiments except experiment 17. Some of the single injections were made into a foreleg vein, the others into a femoral vein. Single injections were made slowly during 30 to 120 seconds, and the time of sampling was measured from the end of the injection. The continuous infusion was made through a cannula introduced into a side branch of the right femoral vein, with its tip just reaching the larger vessel. Physostigmine thus introduced was at once washed into the general circulation. Doses from 0.02 to 62.8 micrograms per kgm. per minute were administered by using various concentrations of the drug injected by a mechanical infusion pump[4] employing a calibrated syringe operating at infusion rates of 2.5 to 20.0 cc. per hour. In each experiment the calculated quantity of fluid injected plus that actually remaining in the apparatus agreed within 2% or less of the total amount prepared.

To attain a definite level of serum cholinesterase activity, a given rate of infusion was maintained and blood samples were taken at half-hourly or hourly intervals until no further drop in cholinesterase activity occurred in two successive samples. The rate of infusion was then doubled or quadrupled, and the procedure was repeated. In this fashion from one to five separate rates of infusion were given to each of sixteen different dogs, starting with the lowest rate of administration in each case (see table 2). The shift from one rate to another was accomplished with only a few seconds' interruption of the infusion. Refilling of the syringe necessitated an interruption of the infusion for not longer than 2 to 3 minutes.

In the experiments with single injections intravenous atropine sulfate was given a few minutes before the physostigmine salicylate to avoid toxic parasympathetic effects. In the continuous infusions these toxic effects were avoided by adding to the physostigmine solution 1 mgm. of atropine sulfate per 20 mgm. of physostigmine salicylate.

In four experiments continuous infusions of physostigmine salicylate were carried out in dogs with the kidneys excluded from the circulation. Before the start of the experiment the kidneys were approached through a midline abdominal incision, mobilized completely, and a double ligature tied around the pedicles. Control blood samples were taken after the abdominal incision was closed. After the experiment the kidneys and pedicles were removed and inspected for leaks. None were found.

1. *The effect of single intravenous injections of physostigmine.* As the relationship between dose and effect with single doses proved to be very complicated, only six experiments in 4 dogs were made. The results are given in table 1; experiment 1 is illustrated in greater detail in figure 1. Experiments 3 and 4 were done on the same dog and on the same day; the larger dose was administered after the serum cholinesterase activity had returned to the normal level. Experiment 5 followed experiment 2 on the same animal after an interval of one week. The experiments, with the exception of 2a, were made with adequate doses of atropine sulfate to protect the animals against the poisonous effect of marked cholinesterase inhibition. In experiment 2a, a dose of only 0.2 mgm. of atropine sulfate was given to the animal intravenously 2 minutes after the end of the physostigmine injection. At this time, when cholinesterase activity was 7.5% of normal the heart rate was still unchanged, 126 per minute; and the respiration rate was 6 per minute (nembutal anesthesia). During the following 3 minutes the animal became cyanotic, and 5 minutes after the end of the physostigmine injection the respiratory rate suddenly increased to 51 per minute and respiration became shallow. The heart rate was 112 per minute. The

cholinesterase activity was now 11% of normal.   Heart beat and respiration stopped simultaneously 8 minutes after the end of the physostigmine injection.

TABLE 1

Dogs in nembutal anesthesia.   The effect of intravenous injections of single doses of physostigmine upon the inhibition of serum cholinesterase activity

	Experiment 3	Experiment 2	Experiment 1	Experiment 4	Experiment 5	Experiment 6
Date of exp	5-20-41	5-16-41	5-16-41	4-4-41	5-20-41	5-23-41
Weight of dog (kgm)	24.6	23.8	21.0	19.3	24.6	23.1
Sex of dog	M	M	F	M	M	M
Physostigmine salicylate (mgm/kgm)	0.10	0.21	0.24	1.04	1.80	2.60
Atropine sulfate (mgm/kgm)	0.04	0.08	*	0.10	0.20	0.22
Serum cholinesterase activity, normal initial values†	9.8	9.4	12.2	6.6	9.8	8.8

Activity of cholinesterase in serum in per cent of normal

Time after injection (minutes)	Exp 3 Obs	Exp 3 Corr	Exp 2 Obs	Exp 2 Corr	Exp 1 Obs	Exp 1 Corr	Exp 4 Obs	Exp 4 Corr	Exp 5 Obs	Exp 5 Corr	Exp 6 Obs	Exp 6 Corr
2	33	11	31.5	10	24	7.5			21	6.5	18	5.5
5	40	14	41.5	15	33	11						
6											22	7
10	52	22	49	21			19	6	21.5	7	19.5	6
30	71.5	47	61	32			24	7.5			27	8.5
50							30	9.5				
60	80	68	73	50					38	13	38	13
110							44	17				
120	92.5	86	91	82.5					43	16	53	23
170							55	25				
180	99.5	99	91.5	84							68.5	42
220							64	36				
240	91	82.5	88	78								
260											88	77
300	94.5	89	99	98								
320											91	82.5
360			96.5	93								
380											94	89
420			106	106								
450											103	103
500											105	105
560											104	104

* Two minutes after the injection of physostigmine a single dose of only 0.2 mgm of atropine sulfate was injected

† These values × 13.5 = millimols of acetylcholine hydrolyzed per liter of serum per hour at pH 7.4 and 38°C, and represent our 100% values

Several points in regard to the experiments of table 1 need amplification. 1) A blood sample obtained 2 minutes after the intravenous administration of 0.1 mgm. and 0.2 mgm. of drug per kgm. of body weight contained enough

of the alkaloid to depress the serum cholinesterase activity to 10% of normal (exp. 2 and 3), while doses 10 and 20 times larger (exp. 1, 4, and 5) depressed the serum activity not much more, to about 5 to 6% of normal. It has been reported (10,11) that there is a relatively slow combination of cholinesterase and physostigmine. The apparent lack of this delay period in these *in vivo* experiments is probably an experimental artifact. Because of the interval necessary for defibrinating the blood, centrifuging the cells, and bringing the serum-bicarbonate mixture to equilibrium, the earliest time at which substrate could be added was at least 35 minutes after the blood sample was taken. Thus, the-
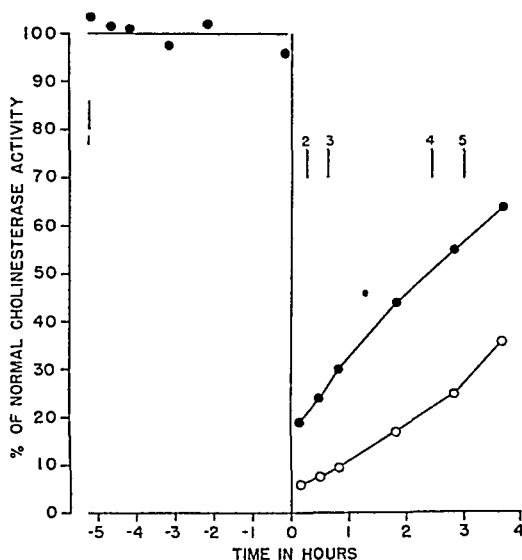
FIG. 1. INHIBITION OF SERUM CHOLINESTERASE ACTIVITY BY A SINGLE DOSE OF PHYSOSTIGMINE SALICYLATE

Dog, male. 19.3 kgm. Nembutal anesthesia. At signal 1, 200 cc. of blood was withdrawn. At zero time, 1.04 mgm. of physostigmine salicylate per kgm. was injected intravenously together with 0.1 mgm. of atropine sulfate per kgm. At signal 2, slight fasciculation of striated muscles appeared. At signal 3, fasciculation was very marked and widespread. At signal 4, fasciculation began to subside, and at signal 5 it had entirely disappeared. ● = observed cholinesterase activity. ○ = corrected cholinesterase activity.

results on samples taken within the first half-hour or more are a good reflection of serum physostigmine concentration, but not of the true inhibition existing *in vivo* at the time when the sample was drawn.

2) The rate of recovery with the dosage range employed is strikingly rapid. As with any substance given intravenously, the shape of a curve relating concentration to time is dependent upon two main factors: (a) There is the initial rapid fall in concentration due simply to distribution of the substance into the extravascular tissues of the body; and (b) the fall in concentration due to excretion, inactivation, or destruction of the drug. It can easily be seen, however,

from figure 1 and from exp 4 and 5 of table 1 that the initial steep rise did not occur with the large doses of physostigmine and that the serum cholinesterase activity stayed for 30 minutes below 10% of normal probably distribution was then complete and recovery from then on was a truer picture of destruction of physostigmine than during the initial part of the recovery curve after the smaller doses Since distribution and destruction presumably start simultaneously, the time action curves plotted from the data of table 1 give a composite picture which is of significance in describing the over all course of recovery but which cannot be used for a detailed analysis of mechanisms of destruction of physostigmine involved

3) The level of serum activity at which various parasympathetic effects began could not be determined since atropine was necessary to prevent death from the doses of physostigmine used, and doses of physostigmine smaller than 0 1 mgm /kgm produced only insignificant and transient changes in serum cholinesterase activity

4) Widespread fasciculation of the striated muscles failed to occur with the smaller doses (exp 2 and 3) and with the larger doses appeared some 10 to 15 minutes after the administration of the drug reached a maximum intensity about 30 minutes later, and disappeared quite uniformly when serum cholinesterase activity rose above 25% of normal during recovery (see fig 1) The tongue and cremaster were always the first muscles to show the onset of this effect When the serum recovered to 25% of normal within 20 minutes, they were the only muscles that showed fasciculation, when, with larger doses of physostigmine, the serum activity at the end of twenty minutes was still below 25%, the larger skeletal muscles then began to fasciculate

2 *The effect of continuous intravenous infusion of physostigmine*   The principal object of this group of experiments was to obtain a steady state of cholinesterase inhibition which would eliminate irregularities due to the unknown factors of destruction and distribution, the magnitude of which is difficult to assess with single doses   We succeeded in attaining levels of cholinesterase inhibition in all of our 16 experiments   The experiment from which figure 2 was derived (no 7 of table 2) illustrates the important details of our procedure   The level of cholinesterase activity expressed in percentage of normal activity was obtained by averaging the last two or three determinations that as a rule showed no marked change at a given rate of infusion   Thus in figure 2 the values at 1, 2, and 3 hours gave a serum level of 31% uncorrected or 10% (corr) at 3 hours the dose was quadrupled, and the values at 4 5, and 6 hours gave an average level of 24% uncorrected, or 7 5% (corr) of normal   A level, once reached, could be maintained for as long as the infusion continued   Attainment of a new level of inhibition going from normal to a lower degree of cholinesterase activity, as was invariably done in our experiments was found to occur within 60 minutes after the change in the rate of infusion   Prompt recovery of serum activity at a rate dependent upon the level of inhibition always began upon stopping the infusion, irrespective of its previous rate or duration

Continuous intravenous infusions were given to 16 dogs of which 12 were

intact, and 4 had both kidneys excluded from the circulation before the start of the infusion. These are summarized in table 2.

Factors without effect upon the cholinesterase level include the depth and duration of nembutal anesthesia, the hematocrit, body temperature, and the administration of enough atropine to prevent the major toxic parasympathetic effects. Even when large single doses of atropine were given, as in experiments
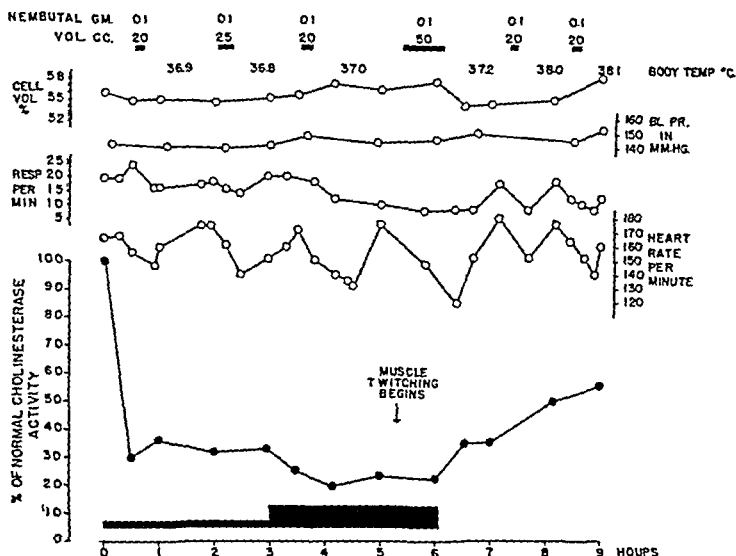
FIG. 2. INHIBITION OF SERUM CHOLINESTERASE ACTIVITY BY CONTINUOUS INTRAVENOUS INFUSION OF PHYSOSTIGMINE SALICYLATE

Dog, female. 21.8 kgm. Nembutal anesthesia. From top to bottom: 1. Nembutal in grams given intravenously during the experiment. 2. Vol. cc. = amount of 0.9% sodium chloride solution administered intravenously together with the nembutal. The horizontal bars below the figures indicate the time taken for the intravenous administration. 3. Rectal temperature in degrees centigrade. 4. Cell vol. = hematocrit. 5. Blood pressure in millimeters of mercury measured with a mercury manometer from the left femoral artery; scale on right. 6. Respiratory rate per min. 7. Heart rate per minute. 8. Serum cholinesterase activity in percent. of normal; scale on left (uncorrected values). 9. Black horizontal bar: From 0 to 3 hours continuous infusion of 3.82 micrograms of physostigmine salicylate and 0.19 microgram of atropine sulfate per kilogram per minute At 3 hours the rate of infusion of the same solution was quadrupled. At 6 hours, infusion was discontinued. For further details see table 2, experiment 7.

2 and 3 (table 2), no effect was noted on the serum level reached; this confirms the observation of Manning, Lang, and Hall (6).

The marked changes in respiratory and heart rates illustrated in figure 2 were correlated with depth of anesthesia. With sufficient atropine present the effect of changes in the level of cholinesterase activity on respiratory rate, heart rate, blood pressure, salivation, vomiting, defecation, and urination was not consistent.

Fasciculation of skeletal muscles, especially the tongue and cremaster, was

TABLE 2

Effect of continuous intravenous infusion of physostigmine upon cholinesterase activity in the blood serum of dogs

I		II		III	IV	V	VI	VII	VIII	IX	X	XI
EXPERIMENT		DOG		PHYSO-STIGMINE SALICYL-ATE	PHYSO-STIGMINE SALICYL-ATE × 10^-9	ATROPINE SULFATE	CHOLINESTERASE ACTIVITY			DURA-TION OF IN-FUSION	TOTAL AMOUNT OF SOLN INFUSED WITH ALKALOIDS	ANES-THESIA*
No	Date	Sex	Weight				Normal CO_2, mM/lt per 20 min	Inhibition % of Normal				
								Ob-served	Cor-rected			
	1941		*kgm*	*micrograms per kgm per min*	*mols per kgm per hr*	*micrograms per kgm per min*				*minutes*	*cc per hour*	
16	11-27	M	27.5	0.02	2.9		6.6	100	100.0	180	5.0	N
				0.04	5.8			95	90.5	180	10.0	
				0.08	11.6			96	92.5	187	20.0	
5	2-14	M	27.2	0.15	21.8		8.2	88	77.5	180	2.5	N
				0.3	43.6			73	50.5	180	5.0	
				0.6	87.1			66	38.5	150	10.0	
6	2-21	F	19.6	0.21	30.5		9.8	89	79.0	165	2.5	N
				0.42	61.0			80	62.0	125	5.0	
				0.85	123.4			67	40.0	125	10.0	
4	2-7	F	25.6	0.65	91.3		7.7	42	15.0	180	2.5	N
				1.3	189.0			35	12.5	180	5.0	
				2.6	377.0			34	11.0	159	10.0	
1	1-17	M	25.0	1.32	192.0		10.2	58	28.5	500	2.5	C
2	1-24	M	25.4	1.3	189.0	(1)	9.8	57	27.5	185	2.5	C
				2.6	377.0			48	19.0	340	5.0	
3	1-30	M	18.0	1.8	261.0	(2)	10.0	51	21.5	300	2.5	C
				3.6	523.0			45	16.5	240	5.0	
9	4-25	M	20.4	0.25	36.3		5.6	84	70.0	180	2.5	N
				0.50	72.6			66	38.5	180	5.0	
				1.98	288.0			48	19.0	180	20.0	
				3.96	575.0	0.25		36	12.0	180	5.0	
				7.92	1150.0	0.50		28	9.0	187	10.0	
8	3-21	M	20.0	1.0	145.2	0.1	9.6	61	32.0	181	2.5	N
				2.0	290.0	0.2		51	21.5	179	5.0	
				8.0	1160.0	0.8		32	10.5	176	20.0	
7	2-28	F	21.8	3.82	555.0	0.19	8.0	31	10.0	180	2.5	N
				15.3	2220.0	0.76		24	7.5	180	10.0	

TABLE 2—*Concluded*

I		II		III	IV	V	VI	VII	VIII	IX	X	XI
EXPERIMENT		DOG					CHOLINESTERASE ACTIVITY					
				PHYSO-STIGMINE SALICYL-ATE	PHYSO-STIGMINE SALICYL-ATE × 10^{-9}	ATROPINE SULFATE	Normal CO_2, mM/l† per 20 min.	Inhibition, % of Normal		DURA-TION OF IN-FUSION	TOTAL AMOUNT OF SOLN. INFUSED WITH ALKALOIDS	ANES-THESIA*
No.	Date	Sex	Weight					Ob-served	Cor-rected			
	1941		kgm.	micrograms per kgm. per min.	mols per kgm. per hr.	micrograms per kgm. per min.				minutes	cc. per hour	
10	5-2	M	20.0	2.5	363.0	0.13	8.7	39	13.5	180	2.5	N
				5.0	726.0	0.25		31	10.0	179	5.0	
				10.0	1452.0	0.50		25	8.0	180	10.0	
				20.0	2910.0	0.50		22	7.0	175	5.0	
				40.0	5810.0	1.00		21	6.5	180	10.0	
11	5-9	F	19.2	3.93	570.0	0.20	11.2	36	12.0	176	2.5	N
				7.85	1140.0	0.39		30	9.5	180	5.0	
				15.70	2280.0	0.79		23	7.0	184	10.0	
				31.4	4560.0	1.58		21	6.5	181	5.0	
				62.8	9120.0	3.15		16	5.0	180	10.0	
13‡	9-13	M	27.8	0.2	29.0	0.01	8.5	79	60.0	342	5.0	N
				0.4	58.1	0.02		(3)		180	10.0	
				0.8	116.0	0.04		60	31.0	224	20.0	
14‡	9-16	M	25.1	0.5	72.6	0.025	10.2	71	46.5	180	5.0	N
				1.0	145.2	0.05		63	34.5	180	10.0	
				2.0	290.0	0.1		50	21.0	178	20.0	
				4.0	581.0	0.2		46	18.0	180	20.0	
12‡	9-9	M	21.2	5.0	726.0	(4)	6.5	34	11.0	180	5.0	N
				10.0	1452.0			24	7.5	180	10.0	
				20.0	2910.0					44	20.0	
15‡	9-19	M	22.0	0.15	21.8	0.007	6.2	82	66.0	181	5.0	N
				0.3	43.6	0.015		76	54.5	180	10.0	
				10.0	1452.0	0.5		28	9.0	180	5.0	
				40.0	5810.0	2.0		14	4.0	178	20.0	

* N = Nembutal; C = Chloralose.

† These values × 13.5 = millimoles of acetylcholine hydrolyzed per liter of serum per hour at pH 7.4 and 38°C.

‡ In experiments 12 through 15 the kidney pedicles were ligated.

(1) Three doses of atropine sulfate, 2, 2, and 0.8 mgm., were given during the last 60 minutes of second period of infusion.

(2) Three doses of atropine sulfate, 2 mgm. each, were given during the last 60 minutes of second period of infusion.

(3) All estimations of this level were lost because of leak in gas analyzer.

(4) 0.5 mgm. atropine sulfate was injected intravenously at minute 104 of first period of infusion. The animal died during the third period.

usually present at some time if levels below 15% of normal serum activity were maintained This effect (see figure 2) commonly appeared some time after a given serum level was reached

The essential data relating rate of infusion of physostigmine salicylate and corresponding percentage of uninhibited cholinesterase activity are assembled in table 2 In figure 3 the values of columns VII and VIII are plotted against the logarithms of the rates of infusion of column IV As can be seen from table 2 and figure 3, the serum cholinesterase activity was determined for infusion
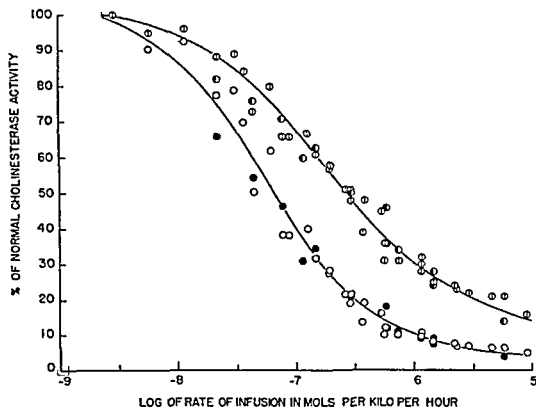
Fig 3 Relation between Rate of Intravenous Infusion of Physostigmine and Percentage Inhibition of Cholinesterase Activity in the Serum of Dogs in Vivo

The logarithm of the rate of infusion, as given in table 2 column IV, is plotted against the level of inhibition as given in table 2 columns VII and VIII ⊕ = observed values ◑ = observed values obtained with kidneys ligated ○ = corrected values ● = corrected values obtained with kidneys ligated

rates over a range of 1 3,000 (0 02 to 62 8 micrograms per kgm per minute), giving activities from 100% down to 5% of normal The standard deviation of points from the solid line (curve of uncorrected figures drawn free hand) is ±3 3% of normal activity, corresponding to ±0 14 of the $\log_{10}$ of the rate of infusion, in the middle portion of the curve This means that one can alter a given rate of infusion in this part of the curve by roughly 25% (and by considerably more at the two extremes) without significantly changing the observed activity The three determinations of experiment 4 are the only values that are out of line and have been excluded from figure 3

The shape of the curve in figure 3 bears consideration At extremely high and

low rates of infusion the slope falls off to zero. In simple terms this is the necessary consequence of the fact that the cholinesterase activity cannot be made greater than normal or less than zero. Most graded physiological responses have this sigmoid form of dose-effect function, but in relatively few reactions is it experimentally possible to explore the whole range *in vivo*.

In agreement with earlier observations by Friend and Krayer (8), column VI of table 2 shows a range of initial serum activities from 5.5 to 11.2 in this series of dogs; no correlation greater than the experimental error could be made between the initial values and the percentage activity achieved with a given rate of infusion of drug.

That there was a reproducible relation between rate of infusion per kgm. and level of serum activity reached in various animals is, in our opinion, the most important result of our investigation. The reproducibility of this function is unusual for experiments *in vivo*, and enables one, we believe for the first time, to reach and maintain a given level of serum cholinesterase inhibition with a predetermined rate of continuous physostigmine infusion. This provides a tool of obvious physiological value in the quantitative study of reactions mediated by acetylcholine.

The exclusion of the kidneys from the circulation did not modify distinctly the level of activity attained with any given rate of infusion. From the urine of the dog given a total of 27 mgm. of physostigmine salicylate in experiment 9 of table 2 we were able to extract material the inhibitory effect of which on serum *in vitro* corresponded to 0.25 mgm. of physostigmine salicylate. This represents a minimum value, since we did not determine the percentage recovery for the method of extraction used. Heubner (13), using physiological assays, and McGeorge (14), measuring cholinesterase inhibition, were likewise able to recover only small fractions of active material from the urine of dogs and human beings respectively. That physostigmine is excreted by the kidney is also shown by the work of Ozawa (15), who reported a ratio for the glomerular:tubular excretion of this drug in the toad. If the kidneys accounted for a significantly large fraction of the total rate of disposal, their exclusion should produce at a given rate of infusion a lower level of serum cholinesterase activity than that obtained in animals with active kidneys. This would be revealed in our method of analysis. Our results, therefore, indicate that the kidneys account for only a minor fraction of the total disposal of physostigmine.

Within the range of infusion velocities studied, i.e., 0.02 to 62.8 micrograms per kgm. per minute, a constant rate of infusion for several hours will decrease the serum cholinesterase activity to a level which remains constant for the duration of the infusion. Under these conditions a steady state is achieved in which the dog must be disposing of the drug at the same rate as it is being administered. We could therefore with equal justice label the abscissa of the graph in figure 3 "rate of destruction" instead of "rate of infusion."

It is obvious from this that the rate of destruction in the animal organism increases with the increase in physostigmine concentration and that the increment in the rate of destruction becomes very great when cholinesterase activity in the serum is decreased below 30% of normal.

TABLE 3

Recovery of cholinesterase activity in the blood serum of dogs after stopping continuous intravenous infusion of physostigmine salicylate

TIME	NO OF EXPERIMENT*													
	5		6		1		2		4		3		7	
	Physostigmine salicylate infused (micrograms per kgm per min)													
	0 6		0 85		1 3		2 6		2 6		3 6		15 3	
	Cholinesterase activity (per cent of normal)†													
	Obs	Corr	Obs	Corr	Obs	Corr	Obs	Corr	Obs	Corr	Obs	Corr	Obs	Corr
minutes														
0	68	44	67	42	58	30	48	20	37	13	41	17	25	7
19							66	40						
30			88	78							55	27	35	12
40							71	49						
60	78	60	90	81	85	72	76	56	55	27	60	32	37	13
90					89	79					70	47		
120	95	90	99	98					67	42			50	21
180	99	98					73	52			56	28		

* For further details see table 2

† The zero time value of cholinesterase activity is the average value (of table 2) for the level of lowest activity
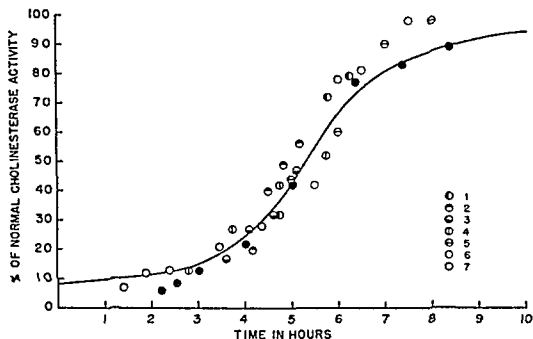
FIG 4 RECOVERY OF CHOLINESTERASE ACTIVITY IN SERUM IN VITRO AND IN VIVO AFTER
INHIBITION BY PHYSOSTIGMINE SALICYLATE

The drawn curve represents the corrected recovery of cholinesterase activity *in vitro* of horse serum inhibited by physostigmine and incubated at 38°C   Circles 1 to 7 = corrected recovery figures of experiments I to 7 of table 3   ● = corrected recovery figures of the X axis refers to the *in vitro* recovery curve
the *in vivo* recovery curve is represented by the position of the first activity
table I starts with the 10 minute value and ends
with the 380 minute value

3. *The recovery of serum cholinesterase activity after inhibition by physostigmine.* In seven of the experiments of table 2 the recovery of cholinesterase activity was followed for up to 3 hours after discontinuation of continuous infusion of physostigmine salicylate. The results are assembled in table 3. They show that recovery begins immediately after stopping the infusion and proceeds at an initial rate which is the slower, the greater the inhibition. In an attempt to correlate the results of the experiments with physostigmine *in vivo* with the observations made on the recovery of the activity of serum cholinesterase from inhibition by physostigmine *in vitro*, we have tried in figure 4 to fit the corrected values of the seven experiments of table 3 to the corrected recovery curve of cholinesterase activity of horse serum inhibited by physostigmine and incubated at 38°C. *in vitro* [see Ellis, Plachte, and Straus (16), figure 4, dotted curve]. For comparison we have also plotted the corrected inhibition values of experiment 5 of table 1, which represents the recovery *in vivo* after the injection of a single dose of 2.6 mgm. of physostigmine salicylate per kgm. The close agreement of the results leads us to assume that the mechanism involved in the recovery *in vivo* after continuous intravenous infusion and after large intravenous single doses may be similar to that *in vitro*.

DISCUSSION

I. *The validity of cholinesterase determinations in inhibited serum.* Any study of the effect of drugs as inhibitors of enzyme systems involves direct or indirect determination of enzyme activity in the absence and presence of the given inhibitor. Such a determination may be based upon measurement *in vivo* of a physiological effect mediated by the enzyme system; or the enzyme itself may be studied *in vitro*, its activity being determined by biochemical methods. One of the enzymes studied most extensively by the latter method is cholinesterase, which is present in serum as well as at specific points of localization in the tissues. The literature is replete with investigations of its properties and functions and particularly its inhibition by compounds related to physostigmine. In the course of such investigations in this laboratory discrepant results obtained by different technical methods led to a thorough analysis of the laws governing the reversible combination of inhibitors with enzymes. It was found that the method of determination of enzyme activity in the presence of an inhibitor has much to do with the degree of inhibition which is observed, and that, in addition to the enzyme concentration and the amount of inhibitor employed, a number of uncontrolled variables are present which must either be eliminated or corrected for, if actual degrees of inhibition *in vivo* are to be evaluated from experimental observations of enzyme activity.

The following discussion is based upon theoretical and experimental work (17) which will be published elsewhere in greater detail. We will summarize briefly what applies specifically to the cholinesterase-physostigmine system and is essential for an understanding of the necessity for and the significance of the corrections made in this paper for observed cholinesterase activity values.

The validity of a serum cholinesterase determination in the presence of an

inhibitor—that is to say, its correspondence to the actual degree of inhibition in the serum of the animal before withdrawal—depends upon the following

1 *Combination* There is evidence, as was mentioned above, that the attainment of equilibrium between physostigmine and cholinesterase may be a slow process Thus serum withdrawn soon after injection of the drug may continue to decrease in activity until the determination is made or equilibrium is reached This effect would be noted in single do e experiments, but not, of course, when a continuous infusion of physostigmine had been maintained Combination should be most rapid with high concentrations of drug and slowest with low concentrations However, since the *total change* at low concentrations is small (e g , from full activity to 96%) it need concern us less than the considerable change taking place slowly at medium degrees of inhibition Thus, inhibition to 80% of normal in serum at the time of withdrawal may change to 70% or less by the time the determination is made, and false low activity will be found This can be confirmed experimentally, but as a matter of fact, even at a serum concentration of 5% at 38°C , combination is complete within two hours, and in whole serum thus equilibrium is reached much more rapidly Therefore, provided there is a delay of an hour or so before determination, this error can be ignored, the usual time spent in preparation of the serum at room temperature and storage in the refrigerator should be ample for this purpose

2 *Destruction* The breakdown of physostigmine in serum and in buffered salt solutions is treated fully in one of the following papers (16) Serum containing inhibitor may be safely kept at 6° for at least 24 hours without change in inhibition At 38° in whole serum the destruction rate is appreciable in the mid range of inhibition and even in 5% serum it is measurable over a period of a few hours An appropriate empirical correction is made for destruction wherever the duration of incubation before determination makes this significant (exp 17, fig 6, and exp 18, table 4 below) In all the experiments described above, however, determinations were made without any lengthy incubation period and no such corrections were applied

3 *Dilution* That dilution of a reversible enzyme inhibitor system leads to dissociation with consequent decrease in inhibition was shown theoretically by Straus and Goldstein (9), who confirmed their conclusions with the cholinesterase physostigmine system This means that the enzyme activity as determined in diluted serum is higher than it actually was in the animal s serum before withdrawal The dilution effect for a given enzyme inhibitor system depends primarily upon the factor of dilution, and to a lesser extent upon the enzyme concentration (i e , enzyme centers) The dilution corrections proposed by these authors were based upon values of enzyme concentration (E) and dissociation constant (K_I) derived by them from experiments with horse serum Although it is clear that the same constants will not apply to dog serum it is a fact that small differences in these constants will affect the dilution corrections but little, It has therefore seemed reasonable to use their figure 3 directly for correction of dat a in the experiments pre ented above It now seems likely that the constants referred to especially the enzyme concentration (E), are somewhat too large

The effect of this error, if significant, will be to make the present dilution corrections too small and our data will be *under-corrected* for dilution.

The work of Straus and Goldstein dealt only with equilibria and did not consider the time required for attainment of the dilution effect. As a matter of fact the dissociation occurring on dilution is a slow effect and significant errors may result if determinations made soon after dilution are fully corrected back to whole serum. Such errors would be in the nature of over-corrections so that the calculated inhibition in the original serum would be greater than was actually the case. The simplest way to avoid this time effect is to allow the serum to stand sufficiently long (incubated at 38°C. and corrected for destruction, or stored at 6°) *after* dilution for the dissociation to be complete (exp. 18, table 4). It is also possible to establish empirical correction curves relating inhibitions at a given time after dilution of a serum-inhibitor mixture to those produced by the equivalent concentration of a drug added directly to diluted serum.

4. Competition. In determining the degree of inhibition of an enzyme by comparison of uninhibited with inhibited enzyme in respect to the rate of destruction of added substrate, one makes the implicit assumption that the substrate itself does not significantly alter the observed inhibition; that is to say that inhibition is non-competitive. As a matter of fact several investigators have reported the *competitive* nature of the system under discussion here. We have confirmed this and have been able to make quantitative studies of the mechanism of this competitive inhibition. When cholinesterase and physostigmine are first allowed to stand together (as in the preceding experiments) and then acetylcholine is added, there is displacement of a certain amount of inhibitor from combination with enzyme even during the twenty-minute period required for the determination. Because of this definite and measurable increase in enzyme activity during the determination, the observed 3-23 minute (after substrate addition) values are always in error on the side of too little inhibition. Unfortunately, the data commonly used for construction of curves which yield values for the constants K and E are themselves subject to this error, so that these constants (as previously mentioned) are actually somewhat too large.

Before addition of any substrate, a typical sigmoid curve exists, relating potential enzyme activity to the logarithm of the inhibitor concentration (NC, Fig. 5). Some time after addition of substrate, when the new competitive equilibrium has been attained, an entirely new curve relates these two variables; this competitive equilibrium curve lies considerably to the right of the first (C, fig. 5). The curve OBS, figure 5, based on 3–23 minute determinations, lies between the other two, since the observed values of enzyme activity have been "caught" in transition between their initial and final values.

There are three legitimate ways to handle the competition effect. All require a reasonably good value for the constants E and K, obtained by methods not employing the inaccurate 3–23 minute curve.

a) The most obvious is to allow sufficient time after addition of substrate and before any reading is made so that the value obtained will represent a point on the competitive equilibrium curve (C). One can then calculate what inhibition

would be produced in absence of substrate by the same concentration of inhibitor, and this point on the non-competitive curve (NC) will be the correct serum inhibition   This method has been used in experiment 18, table 4 below, but it is open to a number of technical objections and greatly prolongs the time required for the determinations

b) The second method is empirical and is useful where, for example, dilution is proceeding as well as competition during the 3-23 minute period, so that separate corrections become impossible   Here an empirical curve is constructed, relating inhibitor concentrations to enzyme activity, and experimental readings
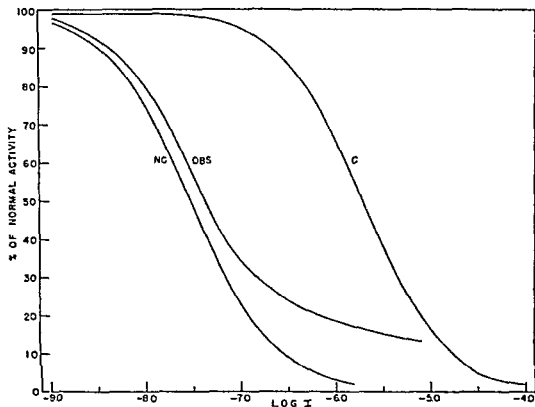
Fig 5 Effect of Competition by Substrate on the Equilibrium Curve Relating Log₁₀ of Inhibitor Concentration in Reaction Mixture to Enzyme Activity
NC = the theoretical non competitive equilibrium curve in absence of substrate   OBS = the curve of observed activity 3-23 minutes after addition of substrate   C = the final competitive equilibrium curve with substrate present   These curves illustrate the principle of competition and are not to be used in correcting the data presented, since they are based upon determinations in 4 51% serum in the Warburg apparatus

are then corrected directly from this curve down to the true non competitive one   This is the procedure we have used in correcting all the data presented above   Unfortunately we could not arrive at an accurate estimate of the value of the enzyme concentration (E), except to place upon it a maximum value of $4\ 0 \times 10^{-7}$ in whole serum   This in turn means that the corrections we have applied to our data by means of the empirical method just described are not entirely accurate   We justify this kind of correction on the grounds that the data are corrected *in the right direction* and to approximately the right extent, and that consequently the corrected activities are certainly nearer to the truth than were

the observed activities.   Further, there is reason to suspect that we have actually *under-corrected* our data and that the true activities in serum are even lower than our figures would indicate.   (These considerations do not apply to experiments 17 of figure 6 and 18 of table 4 below, which are fully corrected as stated under a.)

c) If one knows the function relating time to enzyme activity at various inhibitor concentrations, in a system where competition is proceeding, it is possible to calculate velocity constants which may then be incorporated in a general equation for the correction of 3–23 minute readings back to the time of the addition of substrate.

The following is a tabulation of the errors arising from the various causes discussed:

If combination is not complete when serum is withdrawn, the observed activity is ...   .   *too low*
If destruction is appreciable and not corrected for, the observed activity is..*too high*
If dilution is not corrected for, the observed activity is   *much too high*
If dilution correction is applied fully before dilution equilibrium is attained, the calculated activity is   .   .   *too low*
If competition is not corrected for, the observed activity is   *much too high*

In the future one should be able to avoid most of the corrections entirely and be accurate about the remainder.   Experiments should be done in such a way as to eliminate errors due to combination and destruction; this means that whole serum should be stored in the refrigerator for a few hours after withdrawal.   The error due to slow onset of the dilution effect can be avoided by allowing time for equilibrium to be reached *after* dilution; the full dilution correction will then be applied to the diluted serum-drug mixture.   The competition effect, if present in a given system, cannot be eliminated as long as substrate has to be used for the determination of enzyme activity; but it can be handled satisfactorily by the methods just outlined.

II. *Interpretation of experimental results.* 1. Failure to consider the factors just discussed is undoubtedly responsible for the numerous discrepancies to be found in the literature with regard to the estimation of inhibited activity of cholinesterase even with methods that are based on the same principles as the gasometric methods.   If the discussion just presented is valid, these discrepant values of cholinesterase activity inhibited by the same inhibitor should be brought into harmony if the appropriate corrections are applied.

In experiment 11, part 5, of table 2, 62.8 micrograms of physostigmine salicylate was infused and led to an observed value of 16% of uninhibited activity when the reaction mixture in the Van Slyke gas analyzer contained 22.2% serum.   In a comparable experiment (exp. 17, see fig. 6), 50 micrograms of physostigmine sulfate (corresponding to 63.7 micrograms of physostigmine salicylate) and 2.5 micrograms of atropine sulfate per kgm. per minute were infused and the serum cholinesterase activity was determined with the Warburg constant volume manometer in which the final serum concentration was 4.5%.   The level of cholinesterase activity was approximately 30% of normal (uncorrected).   The

observed values of both experiments correspond to a calculated serum activity of about 5% of the normal when the appropriate correction for the dilution is applied In a similar experiment (exp 18) the corrections were rigorously applied for destruction and competition as well as for dilution after the determinations had been made under carefully controlled conditions   The essential results are shown in table 4   The degree of inhibition is of the same order as in experiment 17, and the recovery values compare favorably with those assembled in table 3

TABLE 4

Exp 18 10/11/43 Dog male 20 5 kgm Nembutal anesthesia   Physostigmine salicylate 60 4 micrograms and atropine sulfate 3 micrograms per kgm per minute infused intravenously for 2 hours Serum stored overnight at 1°C , then diluted to 5 0% and incubated at 38° for 3 hours   0 2 cc 20% acetylcholine bromide added to 2 0 cc of 5% serum and activity observed 3 to 23 minutes thereafter in Warburg constant volume manometer Serum concentration in reaction mixture 4 54%   Normal cholinesterase activity 150 4 mM/liter of serum/hour at pH 7 4 and 38°C

| TIME | | PER CENT OF NORMAL CHOLINESTERASE ACTIVITY | | | |
Absolute	Relative	Observed	Corrected for compett on	Corrected for destruction	Corrected for d lution
a m	*m nu es*				
7 04		93			
7 35		105*			
8 00		101			
8 38		Infusion started			
9 0S		31	20	20	<1
9 3S		42	33	29	2
10 35		35	25	24	1
10 38	0	Infusion stopped			
11 38	60	40	30	28	2
p m					
12 38	120	54	45	41	3
1 38	180	71	63	58	7
3 20	282	76	70	65	9
4 00	322	88	85	82	26
4 53	375	90	88	85	33
6 01	443	92	90	87	39

* Average 100%

The continuous infusion data with physostigmine salicylate obtained by Rentz (7) in cats compare fairly well with our results in dogs   It has to be kept in mind, however, that his period of infusion of each dosage was only about 20 minutes, a time which is probably insufficient to bring the animal to a state of dynamic equilibrium, especially with low dosages   In table 5 we have assembled the essential data taken from figure 7 of Rentz' paper   His observed percentage inhibition of serum cholinesterase activity, obtained from reaction mixtures containing 7% serum, were corrected and the corrected values of serum inhibition compared with the expected corrected percentage inhibition values of dog serum

from our figure 3.   (In none of our experiments in dogs did we observe, as Rentz did in one of his cat experiments, an increase in cholinesterase activity upon infusion of physostigmine.)

A single intravenous injection of 0.29 mgm. per kgm. of physostigmine sulfate (equivalent to 0.37 mgm. of physostigmine salicylate) was given to a dog by Ammon and Voss (4).   Twenty minutes after the injection the serum cholinesterase activity was reported to have been 47% of the normal.   As the analysis was done by the Warburg method in 1.125% serum the corrected activity of the undiluted serum would have been about 2 to 3%.   This is lower than the value of about 7% in our experiment no. 1 of figure 1 and table 1, in which a dose of 1.04 mgm. of physostigmine salicylate per kgm. was given intravenously.   Part of the discrepancy in the corrected inhibitions probably is to be ascribed to the considerable inaccuracy of the determination of the activity of an inhibited serum when the dilution is as great as 1:89, and also to the uncorrected time effect between dilution and determination.

TABLE 5

Relation between rate of intravenous infusion of physostigmine salicylate and inhibition of serum cholinesterase activity

RATE OF INFUSION	RENTZ' FIG. 7 (CAT), % NORMAL ACTIVITY		EXPECTED CORRECTED % OF NORMAL ACTIVITY FROM FIGURE 3 (DOGS)
	Determined	Corrected	
micrograms per kgm. per min.			
0.5	91	62	40
0.7	72	19	15
5.0	52	7	10
50.0	52	7	5

2. It was mentioned in the introduction that Ammon and Voss used a dose of 5 mgm. of physostigmine sulfate in their 17 kgm. dog, because this corresponded to the dosage used in the experiments of Feldberg and Krayer (2) and Krayer and Verney (3).   It is of interest in this connection to emphasize that these authors had difficulty in demonstrating an increase in the concentration of acetylcholine in the coronary blood upon vagal stimulation after one single dose of physostigmine sulfate of 5 to 10 mgm., while repeated doses gave satisfactory results. This is undoubtedly due to the rapid recovery of cholinesterase activity after a single dose of this size and not to an insufficient degree of initial inhibition of cholinesterase as the uncorrected value of Ammon and Voss (47% of normal activity) might well suggest.   The appropriate way of administering physostigmine for investigations of changes in acetylcholine concentration obviously is the continuous infusion of physostigmine as shown in figure 6, which is a record of an experiment that, except for the continuous infusion of physostigmine and the estimation of cholinesterase inhibition, in every other respect is similar to those of Feldberg and Krayer.

The dog of experiment 17, figure 6, was given artificial respiration.   100 mgm. of heparin was injected to prevent clotting of the blood.   A Morawitz cannula was introduced into

the coronary sinus The vagus nerves were severed in the neck and the peripheral end stim
ulated by a strong tetanizing current from a Harvard inductorium The eserinized leech
muscle was used to identify acetylcholine Immediately before adding coronary blood to
the leech muscle it was diluted 1 2 with cold Tyrode solution, acetylcholine concentration
in blood therefore, is twice as high as indicated by the comparison of the leech muscle re
sponses caused by blood with the corresponding calibration curves The tracings of the
leech muscle reactions were placed on the graphical record of changes in cholinesterase
activity in figure 6 such that the time of addition of blood to the leech muscle corresponds
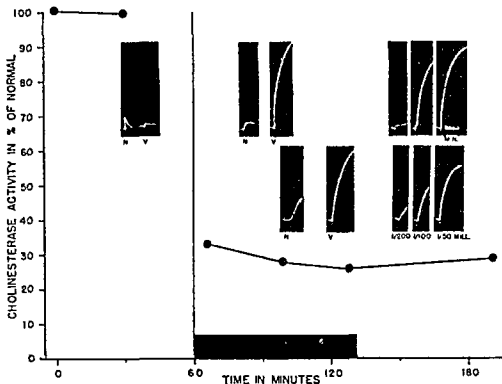
FIG 6 EXPERIMENT 17 EFFECT OF PERIPHERAL VAGAL STIMULATION UPON THE ACETYL
CHOLINE CONCENTRATION IN THE CORONARY BLOOD AT NORMAL AND AT INHIBITED
SERUM CHOLINESTERASE ACTIVITIES

Dog male, 18 kgm Nembutal anesthesia Vagi cut Morawitz cannula in coronary
sinus The black bar indicates the duration of physostigmine and atropine infusion
Cholinesterase activity was determined with the Warburg constant volume manometer,
the final serum concentration was 4 54% The percentage values of cholinesterase activity
are uncorrected At N normal blood and at V blood obtained during vagal stimulation
were added to one (upper) or the other (lower) leech muscle strip suspended in Tyrode
. . C
curve Acetylcholine bromide (mol weight 226) contains 63% acetylcholine Concen
trations of acetylcholine correspond to ⅔ of the acetylcholine bromide concentrations

to the time value for cholinesterase activity present at the moment when the blood was
taken from the coronary cannula Physostigmine infusion started at 60 minutes and was
discontinued at 131 With normal activity of cholinesterase in the serum even during vagal
stimulation the acetylcholine concentration in the coronary blood was below 1 150 million,
after a thirty minute infusion of physostigmine vagal stimulation increased the acetyl
choline concentration from about 1 150 million to 1 35 million (see N and V, upper leech
tracings) 10 minutes later it was still more than 1 110 million (lower leech tracing N) and
again rose to more than 1 35 million upon another vagal stimulation (lower leech tracing V)
For further details concerning the method used in this experiment see (2)

This experiment from another point of view emphasizes the necessity of correcting observed inhibition of cholinesterase activity in diluted serum, in that it is difficult to understand how an acetylcholine concentration between 1:35 million and 1:100 million should be present for several minutes in blood with a serum cholinesterase activity still as high as 30% of normal, while the corrected values, revealing nearly complete inhibition, resolve this difficulty.

3. The clinical importance of this correction can be well illustrated with the data obtained by Jones and Tod (5). The cholinesterase activities of their human serum samples were determined manometrically in a final reaction mixture containing a dilution of 1:15 of the original serum. In all but one patient a definite inhibition after the subcutaneous injection of physostigmine sulfate was noted which ranged from 73 to 85% of the value before administration of the drug. When the dilution effect is taken into consideration the corrected values of inhibited serum cholinesterase activity ranged between 21 and 45% of the uninhibited activity.

4. There are some discrepancies between our results and those of Manning, Lang and Hall (6). The recovery of serum cholinesterase activity after single doses of physostigmine in our table 1 and figure 1 not only had a different time course, but the recovery periods even with the small doses were considerably longer than Manning, Lang and Hall observed. In an unanesthetized dog (see their graph 1) with the dose of 0.1 mgm. of physostigmine sulfate per kgm., normal activity had returned within 46 minutes after the injection, and within 17 minutes after the maximum inhibition had been reached.

These authors reported a profound inhibition of serum cholinesterase activity without more than slight parasympathetic effects when 0.05 mgm. of physostigmine sulfate per kgm. in 50 cc. of saline per half-hour was infused continuously into unanesthetized dogs. Cholinesterase activity in serum was determined by the continuous titration method of Hall and Lucas (18). In the experiment of table 4 of Manning, Lang and Hall the infusion of 1.66 micrograms of physostigmine sulfate per kgm. body weight per minute in a dog of 19 kgm. caused on the first day of the experiment a decrease to 34% (uncorrected) of normal activity of serum cholinesterase. This dose is equivalent to 2 micrograms of physostigmine salicylate, and corresponds to the dose given in experiment 8, part 2, and experiment 9, part 3 of our table 2. The corresponding inhibition is 50% uncorrected and 20% corrected. We were able to observe definite and consistent parasympathetic effects at the level of cholinesterase activity reached with this dose with regard to the effect upon the gastro-intestinal tract. The results of those experiments of table 2 in which physostigmine was infused without atropine are given in table 6. (No graphical records were taken, but repeated emptying of the bowels was taken as indicating increased intestinal activity. No accurate measurements were made of salivary flow.)

As is evident from table 6 increase in intestinal activity resulting in defecation occurred with certainty only if cholinesterase activity in the serum decreased to 40% of normal (corresponding to an infusion of approximately 0.5 microgram of physostigmine salicylate per kgm. per minute); while even slight salivation in

these anesthetized dogs could not be detected at a serum cholinesterase activity above 25% of normal (corresponding to an infusion of approximately 1 5 micro grams of physostigmine salicylate per kgm per minute)

5 The delay period between the administration of physostigmine in a single dose or by continuous infusion and the appearance of fasciculation of striated muscles demands an explanation   A correlation of fasciculation with the serum potassium rather than the cholinesterase activity after doses of prostigmine was reported by Thompson and Tice (19)   We have not made serum potassium esti- mations in our physostigmine experiments, and our data seem to give weight to

TABLE 6

Dogs in anesthesia (N = nembutal C = chloralose)   Continuous infusion of physostigmine Relation between level of serum cholinesterase activity and effect on intestinal activity and salivation

EXP NO *	SERUM CHOLINESTERASE ACTIVITY % OF NORMAL†		INTESTINAL ACTIVITY	SALIVATION	ANESTHESIA
	Obs	Corr			
16	100	100	No effect	None	N
16	96	92 5	No effect	None	N
6	89	79	No effect	None	N
5	88	77	No effect	None	N
9	84	70	No effect	None	N
6	80	62	Increased	None	N
5	73	50 5	No effect	None	N
6	67	40 0	Increased	None	N
9	66	38 5	Increased	None	N
5	66	38 5	Increased	None	N
1	58	28 5	Increased	None	C
2	57	27 5	Increased	None	C
3	51	21 5	Increased	Slight	C
9	48	19 0	Increased	Slight	N
2	48	19 0	Increased	Marked	C
3	45	16 5	Increased	Marked	C

* For further details see table 2

† From columns VII and VIII of table 2

the correlation of muscle effects with serum cholinesterase activity The delay in appearance of the fasciculation may well be due to the time necessary to build up sufficient acetylcholine to stimulate the muscle fibers (12)

We do not think that this delay is due to the slow combination with cholinester ase at the nerve muscle junction, since experiments of Brown, Dale and Feldberg (20) and Rosenblueth and Morison (21), as well as from our own laboratory (22), show that in isotonic records from the freed Achilles tendon in cats with supra- maximal stimulation of the peripheral end of the sciatic nerve at the rate of 1 per 4 to 10 seconds, the contraction increases markedly within the first ten minutes after a single intravenous dose of about 0 1 mgm per kgm of physostigmine salicylate and soon decreases to a level somewhat above the normal

SUMMARY

1. The time course of the inhibition of serum cholinesterase activity has been followed in detail after single intravenous doses of physostigmine in dogs.

2. With continuous intravenous infusions of physostigmine in dogs a steady level of inhibited serum cholinesterase activity is reached within one hour and may be maintained for as long as the infusion lasts.

3. The percentage level of serum cholinesterase inhibition attained by a constant rate of infusion is reproducible and allows quantitative definition of "escrinization" when employed for physiological experiments involving cholinergic mechanisms.

4. The kidneys are proved to play a minor rôle in the disposal of physostigmine.

5. The recovery of serum cholinesterase activity *in vivo* after continuous infusion and after large single doses appears to follow a mechanism similar to the recovery of the inhibited enzyme activity *in vitro*.

6. The validity of determinations of inhibited enzymes is discussed from the standpoint of the various methods which may be employed in such determinations. Combination of inhibitor with enzyme, destruction of inhibitor, dilution, displacement of inhibitor by substrate, and time are shown to be among the factors by which methods differ, and which require proper correction. A basis is provided for understanding and evaluating the unfamiliar corrections applied to the data presented in this paper.

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METHEMOGLOBINEMIA AFTER ADMINISTRATION OF p-AMINO-ACETOPHENONE AND p-AMINOPROPIOPHENONE

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The demonstration by Evelyn and Malloy (1), based on the studies of Drabkin and Austin (2), that the degree of methemoglobinemia may be accurately estimated and distinguished from sulfhemoglobinemia by means of the spectrophotometer affords an opportunity to reassay methemoglobin-forming drugs from the standpoint of dosage and response. This has recently been reported for acetanilide and acetopheneditin by Lester (3), (4), who carefully assayed and evaluated the responses of various species to these drugs. Our interest in this subject was aroused by the production of an extreme degree of methemoglobinemia by one of a series of experimental drugs. Since the drug is an isomer of acetanilide, it was considered desirable to compare these new methemoglobin-formers with other known potent compounds having this property.

Chemical. The compounds studied belong to the group of phenones and are:

p-amino-acetophenone   p-amino-propiophenone   p-amino-benzophenone
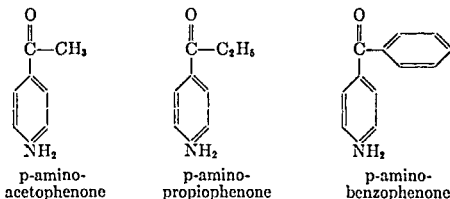
The compounds p-dinitrobenzene, p-aminophenol and aniline HCl were used as controls for purposes of comparison since Issekutz (5) found the first to be active at 1 mgm /kgm subcutaneously in the cat and since p-aminophenol is one of the excretion products of acetanilide reported to be more active than the parent compound.

EXPERIMENTAL Dogs which had been starved for 16 hours were used as test animals The drugs were administered either intravenously or orally in gelatin capsules For intravenous administration, the phenones and aniline were dissolved in dilute hydrochloric acid, while p dinitrobenzene and p aminophenol were dissolved in 50% propylene glycol Dosage was expressed in mgm /kgm Control and experimental blood samples were taken from the jugular vein using 0 25 cc of an oxalate anticoagulant[2] for each 5 cc. of blood.

A Beckman model DU quartz spectrophotometer with 1 cm. cells was used in all determinations. Samples were examined following an adaptation of the method described by Evelyn and Malloy (1). In this method, the extinction of a methemoglobin absorption band at λ630 mμ is measured. Upon the addition of sodium cyanide the methemoglobin is changed to cyanmethemoglobin with disappearance of the band. From the decrease in extinction, the amount of methemoglobin originally present is determined.

Half a cc. of fresh control blood containing anticoagulant is diluted to 10 cc. with M/60 (pH 6.6) phosphate buffer. After allowing 3 to 5 minutes for complete hemolysis, the extinction at λ630 mμ is read. Upon the addition of 1 drop of 10 per cent sodium cyanide solution (freshly neutralized with an equal volume of 12 per cent glacial acetic acid), no significant change is effected if the sample is free from methemoglobin.

With unknown samples, the extinction of a 3 cc. aliquot of a dilution prepared as above is read at λ630 mμ. One drop of sodium cyanide solution is added and the extinction read for the solution now containing cyanmethemoglobin. The difference between the two values is the change due to conversion of the methemoglobin to cyanmethemoglobin. Similarly, control readings are obtained for the same blood samples by quantitatively converting another 3 cc. aliquot to 100 per cent methemoglobin with one drop of 5 per cent potassium ferricyanide solution. The extinction is then read. Following the addition of sodium cyanide, the extinction is read again. The percentage of methemoglobin in the unknown is then calculated from the formula:

$$\frac{\text{Drop in extinction (at λ630 mμ) of sample}}{\text{Drop in extinction of sample changed to MHb}} \times 100 = \% \text{MHb}$$

Due to the anoxic rise in absolute hemoglobin concentration, it was found that the relative percentage methemoglobin is a more accurate index of methemoglobinemia than is the expression of methemoglobinemia as grams of methemoglobin present per 100 cc. of blood. Analysis of the data obtained from all dogs in which the methemoglobin was above 50% revealed that the anoxic rise in hemoglobin ranged from 10 to 20%.

RESULTS.   Tables 1 and 2 and their legends summarize the experimental data obtained following oral administration, and table 3 gives additional data on the effect of intravenous administration of the most important drugs. Compared to the aliphatic phenones, p-aminobenzophenone was found to be inactive.

The signs and symptoms observed in the dogs as a result of extreme degrees of methemoglobinemia were: ataxia at 60% methemoglobin, salivation and prostration at 75%, loss of consciousness at 85% and death at 95% (with p-aminopropiophenone). In the prelethal period respiration was characterized by rapid panting with interspersed single maximal inspirations. Death was apparently due to a gradual diminution in cardiac action.

Figure 1 is a comparison of the visible absorption curves of methemoglobin produced in vitro with potassium ferricyanide and that produced in the dog with p-aminoacetophenone (table 1—Dog No. 200—4/19/43—45 minutes). They are identical, indicating that only methemoglobin is formed. The absence of an absorption band at λ620 mμ indicates that no sulfhemoglobin is present.

Since p-aminoacetophenone is approximately 10 times more active in forming methemoglobin than its isomer acetanilide (3) (4), the question of possible contamination of acetanilide with p-aminoacetophenone was investigated. In present manufacturing methods a chemical rearrangement might occur in the presence of a slight amount of zinc as an impurity. Spectrophotometric analysis

TABLE 1

Experimental drugs—orally

A maximum of 94% to 95% methemoglobin was reached in three dogs before death occurred   In five dogs the methemoglobin attained a peak of 82% to 87% with recovery Dog #131 recovered from p aminopropiophenone methemoglobinemia in spite of a level of 80% which was maintained for 24 hours   All dogs given doses from 2 mgm /kgm to 30 mgm /kgm of p aminoacetophenone recovered   Three of six dogs lived at a dose of 60 mgm /kgm of p aminoacetophenone   Methemoglobin determinations were made on two of these six dogs   Of five dogs given a dose of 50 mgm /kgm of p aminopropiophenone only two lived   p Aminobenzophenone was inactive at 10 mgm /kgm

DOSE	DATE	DOG NO	CONTROL SAMPLE	METHEMOGLOBIN (%)										REMARKS
				1 hr	2 hrs	3 hrs	4 hrs	5 hrs	6 hrs	7 hrs	24 hrs	48 hrs	72 hrs	
p aminoaceto phenone														
2 mgm /kgm	3/22/43	131	0	1 1	7	6				1 1				
10 mgm /kgm	3/17/43	40	2o	23	33	35	37		34					
10 mgm /kgm	3/29/43	81	0	0	20			40	31					
30 mgm /kgm	3/17/43	55	0	31	47	57	57	50	43					
60 mgm /kgm	3/22/43	55	0		87									Dog recovered
60 mgm /kgm	3/22/43	40	0		72									"
(minutes)				45 m n	75 m n	110 m n	170 m n	130 m n	150 m n	210 min	360 m n	24 hr		
70 mgm /kgm	4/19/43	200	0	64	78	83	83	84	83	84	74	13		"
p aminopropio phenone														
10 mgm /kgm	3/22/43	81	0	56	82		77		35					
10 mgm /kgm	3/29/43	00	0	0 6	2 9		54		51					
50 mgm /kgm	3/30/43	55	0			94								Died in 3½ hrs
50 mgm /kgm	3/30/43	131	0			83	85				82	30	13	Dog recovered
50 mgm /kgm	3/30/43	28	0		82*									Died in 2 hrs
50 mgm /kgm	3/30/43	00	0		82	74						2 5		Dog recovered
(minutes)					90 m n		105 m n	110 m n						
50 mgm /kgm	4/ 1/43	70	0		88		95	78*						Dog died
60 mgm /kgm	4/ 7/43	100	0	91	95									Died in 2 hrs
p aminobenzo phenone														
10 mgm /kgm	3/22/13	40	0	0	0			0						

* Blood sample from heart of dead dog (clotted)

TABLE 2

Control drugs—orally

The control amines, aniline hydrochloride and p-aminophenol when compared on a dosage basis are relatively inactive. The most active nitro-compound, p-dinitrobenzene, approximately equals p-aminopropiophenone in activity.

DOSE	DATE	DOG NO.	METHEMOGLOBIN (%)							REMARKS
			1 hr.	2 hrs	3 hrs	4 hrs	5 hrs	6 hrs	7 hrs	
Aniline HCl										
10 mgm./kgm.	3/17/43	131	0	0.5	0.7	0.3	0.2		0.2	
10 mgm./kgm......... .	3/22/43	55	2	0.3	0					
30 mgm./kgm..	3/17/43	81	6	17	14	12	11		5	
p-dinitrobenzene										
10 mgm./kgm.	3/29/43	131	8	27		54		67		
40 mgm./kgm. ..	4/ 1/43	100				83			55	Dog recovered
p-aminophenol										
10 mgm /kgm.	3/29/43	28	8	3		2		2		

TABLE 3

Methemoglobin from intravenous administration

Given intravenously, p-dinitrobenzene acts immediately to produce a maximum of 26% methemoglobin in 7 minutes. This maximum effect decreases gradually. p-Aminoacetophenone, p-aminopropiophenone, and p-aminophenol cause a maximum rise in methemoglobin in 30–60 minutes. p-Aminopropiophenone given intravenously produces more methemoglobin than the acetophenone. p-Aminophenol is again relatively inactive. The mean effect from 1 mgm./kgm. of p-aminopropiophenone about equals that from p-dinitrobenzene, although the peak of action is delayed and hence the mode of action may be different.

DRUG	DATE	DOG NO	CONTROL SAMPLE	MINUTES AFTER INJECTION							
				7	15	30	60	90	120	180	240
p-aminoacetophenone											
2 mgm /kgm.	3/22/43	28	0	15	20	27		21	11		7
1 mgm /kgm.	4/ 5/43	120	0		3 3	6	7.5		7	5	
p-aminopropiophenone											
1 mgm./kgm.	3/29/43	85	0	29	38	41	45	45		34	
1 mgm./kgm.	4/ 5/43	53	0		18	19	20		18	12	7
p-aminophenol											
1 mgm/kgm	4/ 5/43	50	0		1 2	1 4	0		0	0	
p-dinitrobenzene											
1 mgm./kgm	4/ 5/43	81	0 3	26	26	23	17		14	7	3

(fig. 2) indicated that the sample of acetanilide U S P. tested was not contaminated as much as 1% by this isomer.

The toxicity of these compounds (table 4) was determined in the albino rat after intraperitoneal administration. A minimum of forty rats in groups of
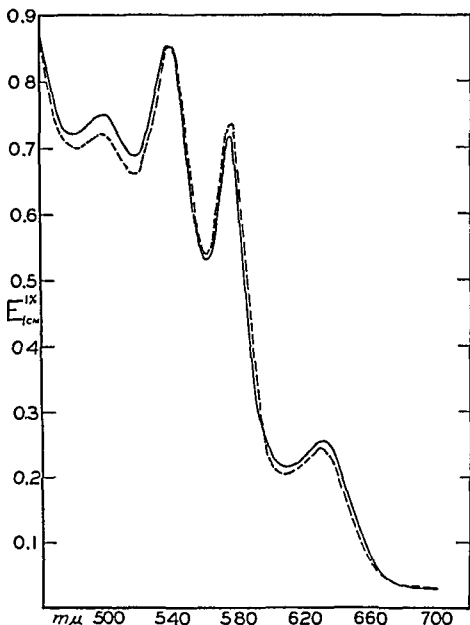
FIG 1 VISIBLE ABSORPTION CURVES SHOWING IDENTITY OF METHEMOGLOBIN PRODUCED IN VITRO BY POTASSIUM FERRICYANIDE AND THAT PRODUCED IN VIVO BY p-AMINOACETOPHENONE

Solid line 64% MHb in vivo
Broken line. 64% MHb in vitro.
Each curve represents approximately 60 points, all of which lie directly under the lines

ten at pertinent dosage levels was used to determine the LD 50 for each compound according to the method of Trevan (6) The rat LD 50's probably reflect the dog data in that death in the rat is due to methemoglobin formation It is interesting that p dinitrobenzene and p aminopropiophenone are equally toxic
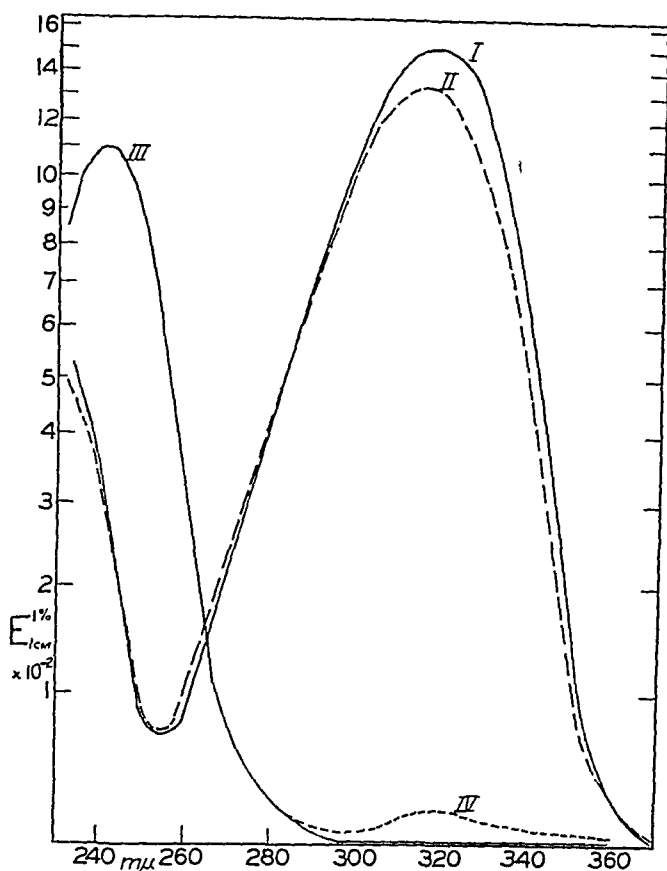
FIG. 2. ULTRAVIOLET ABSORPTION CURVES OF p-AMINOACETOPHENONE (CURVE I),
p-AMINOPROPIOPHENONE (CURVE II), ACETANILIDE (CURVE III), AND
ACETANILIDE CONTAINING 1% p-AMINOACETOPHENONE (CURVE IV),
ALL IN ABSOLUTE ETHYL ALCOHOL

The presence of 1% p-aminoacetophenone in acetanilide can be readily detected spectro-
photometrically. This indicates that the sample of acetanilide U.S.P. employed was not
appreciably contaminated by its isomer p-aminoacetophenone.

TABLE 4

Acute toxicity of methemoglobin-producing compounds

	ANILINE HCl	p-AMINO-PHENOL	p-DINITRO-BENZENE	p-AMINO-ACETO-PHENONE	p-AMINO-PROPIO-PHENONE
L.D. 50 (125 gm. male rats), mgm./kgm. I.P..................	460	465	56	260	55

DISCUSSION   The high prelethal degree of methemoglobinemia produced in the dog by p aminopropiophenone indicates that the compound is probably not toxic other than by way of the anemic anoxia resulting from the inactivation of the hemoglobin   These deaths with 95% of the hemoglobin inactivated can be compared with the deaths in human beings at 77–89% CO—hemoglobin saturation reported by Nicloux (7) and the deaths in the dog at 60–70% methemoglobin saturation after acetanilide treatment reported by Dennig (8)   In the case of carbon monoxide poisoning, the deaths result at a lower level because of the known 'double action" of CO—, namely, inactivation of hemoglobin, and the prevention of O_2 release from hemoglobin (9)   In the case of acetanilide, the large dosage needed to produce methemoglobinemia probably results in cardiac depression (10) and hence a reduced ability to survive extreme anoxia   The severity of the symptoms encountered in the range of 70 to 95 per cent methemoglobinemia is probably out of proportion to those symptoms which arise from inadequate oxygen transport due to a slow progressive anemia   The study of Darling and Roughton (11) on the dissociation curves of methemoglobin indicate that methemoglobinemia, like carbon monoxide poisoning (but to a lesser degree) interferes with the liberation of oxygen from hemoglobin (12)   Some degree of this double action may account for the severity of the observed symptoms which are not, however, as severe as those reported in carbon monoxide poisoning for the same degree of hemoglobin inactivation

The extreme degree of methemoglobinemia obtained with p aminopropiophenone is, from the standpoint of dosage, comparable only to that of the nitrobenzenes   Since the compound is easily soluble and not otherwise toxic it may provide a tool for the study of anoxia   Although these phenones are active they do not act immediately   We were not able to produce methemoglobin with either of these compounds when they were added to blood *in vitro*   They have a more prolonged action than nitrobenzene and the anemia which frequently occurs after the use of nitrobenzene compounds is not observed   For purposes of producing methemoglobinemia they are superior to nitrites and chlorates in that no other pharmacological action obtains   The data also indicate that only methemoglobin and not sulfhemoglobin is formed

SUMMARY

The compounds, p aminoacetophenone and p aminopropiophenone, whether given orally or intravenously, are highly active in forming methemoglobin   The latter is the more active, being as active as p dinitrobenzene, but differing in that its effect is less rapid and more prolonged   Death occurs at 95% methemoglobinemia and recovery may occur with 87% methemoglobinemia   Due to the desirable chemical and pharmacological properties of p aminopropiophenone this compound may prove of value for the experimental production of methemoglobinemia and anoxia

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PHARMACOLOGIC ACTION OF ERYTHRINA ALKALOIDS

I β ERYTHROIDINE AND SUBSTANCES DERIVED FROM IT

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Erythrina L., a genus of trees and shrubs, (rarely perennial herbs) with showy flowers of various shades of red, is widely distributed over the tropics and sub tropics of the entire globe (1). In Central America species of *Erythrina* have played an important role in the folk medicine of pre conquest times. In Mexico, according to Standley (2), decoctions of various parts of these plants are said to have purgative, diuretic or sudorific effects, the poisonous, bean like seeds are used as a hypnotic. Apparently, the first experimental study of extracts of seeds of *Erythrina* is that of Dominguez and Altamirano (3) who, in 1877, observed their paralyzing action in animals. Subsequent sporadic and con tradictory statements on pharmacologic effects of *Erythrina* seeds are recorded in the literature (4). Only recently, the curarizing action of the seeds has been reestablished by Ramirez and Rivero (5), Lehman (6), and Cicardo and Hug (7), and a systematic study of the curare like activity of seeds of 51 species of *Erythrina* has been reported by Folkers and Unna (8).

Chemical research by Folkers, Koniuszy and Shavel on the active principles of *Erythrina* resulted in the isolation of numerous alkaloids from the seeds of various species of *Erythrina*. Erythroidine, the first alkaloid to be isolated from *Erythrina americana* Mill (9) was shown to consist of a mixture of at least two isomeric alkaloids which are dextro rotatory and were named α and β-erythroidine (10). Since the β form is more readily obtained in pure state, chemical and clinical investigations have centered on this substance. In con tinuation of previous studies (11) this paper presents data on the pharmacologic action of β erythroidine, dihydro β erythroidine, β tetrahydro β erythroidine and of other substances derived from these alkaloids

β Erythroidine is a tertiary ammonium base of the empirical formula $C_{16}H_{19}NO_3$ which reacts with methyl iodide to give a methiodide $C_{17}H_{22}NO_3I$ (10). β Erythroidine hydrochloride is a crystalline salt freely soluble in water. Several hydrogenated derivatives of β-erythroidine have been prepared (12). Of these, dihydro β erythroidine hydrobromide and β tetrahydro β erythroidine hydrobromide have been established as pure compounds, α tetrahydro β erythroidine hydrobromide was obtained essentially pure. Because β erythroidine and its hydrogenated derivatives contain a lactone group their reaction with sodium hydroxide solution yields the sodium salt of the corresponding hydroxy acids

The substances used in this study were obtained through the courtesy of Dr. Karl Folkers. β Erythroidine and dihydro β-erythroidine were available in

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the form of their hydrochloride and hydrobromide respectively, α-and β-tetra-hydro-β-erythroidine as hydrobromides. The solutions of sodium β-erythroidinate and of sodium dihydro-β-erythroidinate used in these experiments were carefully freed from excess alkali.

TOXICITY. Acute toxicity following oral and subcutaneous administration was studied in mice, rats, rabbits and cats. A total of 300 mice, 320 rats, 47 rabbits and 27 cats were used. The results are summarized in table 1. The toxicity in mice and rats expressed as the L.D. 50 was determined by using 10 animals for each dose level. The number of experiments with cats and rabbits was too small to allow the calculation of the L.D. 50 with reasonable accuracy; therefore, the toxicity in these species is summarized by giving the dose range

TABLE 1

Lethal doses of erythrina alkaloids

	MICE		RATS		RABBITS		CATS	
	No. of animals	L.D. 50	No. of animals	L.D. 50	No. of animals	L.D. range	No. of animals	L.D. range
		mgm./kgm.		*mgm./kgm.*		*mgm./kgm.*		*mgm./kgm.*
β-Erythroidine HCl								
Oral.............	50	75.0	80	510.0	8	200–400	6	30–40
s.c..............	50	48.0	60	1260.0	10	50–80	8	20–25
Dihydro-β-erythro-idine HCl								
Oral.............	70	7.5	60	320.0	16	30–60	4	2–3
s.c..............	50	9.3	120	230.0	13	10–20	9	2–3
β-tetrahydro-β-erythroidine HBr								
s.c..............	40	9.5						
Sodium β-erythro-idinate								
s.c..............	40	230.0						

in which deaths have been observed, corresponding roughly to the range between the L.D. 1 and the L.D. 100.

Dihydro-β-erythroidine is between 2 and 10 times more toxic than β-erythroidine. β-tetrahydro-β-erythroidine has the same toxicity in mice as dihydro-β-erythroidine, whereas sodium β-erythroidinate is much less toxic than β-erythroidine.

As evidenced in table 1, the sensitivity of different species to these alkaloids varies considerably. Lethal doses in mice coincide roughly with those in rabbits. Rats are much more resistant, the L.D. 50 being about 10 times greater than that in mice and rabbits. On the other hand, cats are more sensitive to the *Erythrina* alkaloids than the other species. A few experiments in dogs indicate that toxic doses in dogs approximate those in cats.

The alkaloids are rapidly absorbed from the gastro-intestinal tract as evidenced by the rather small difference between the oral and subcutaneous lethal doses.

In some species, the oral administration is even more effective than subcutaneous injection (table 1) Furthermore, the toxic effects following oral and sub cutaneous administration becomes manifest at about the same time In cats for instance, the lethal dose range of dihydro β erythroidine is the same following oral or subcutaneous administration, 3 mgm /kgm of this alkaloid caused death after 20 minutes, irrespective of the mode of administration

The toxic manifestations of β-erythroidine and its dihydro derivative were essentially the same All animals died apparently of peripheral respiratory failure, the heart continued to beat for several minutes after the respiration had ceased In mice and rats most of the deaths occurred within one hour after subcutaneous or oral administration of the alkaloids Shortly after reaching the paralytic stage, diaphragmatic breathing became laboured and irregular and the animals died of asphyxia In rabbits and cats the course of toxic manifesta tions observed was more gradual The first sign was inability to hold up the head The animals became very quiet, were unable to stand and lay on their side The respiration became diaphragmatic, but continued to be slow and regular for periods of 10 to 20 minutes In many instances the pupils were widened but reacted promptly to light The heart was beating regularly even after the breathing became irregular or had stopped entirely There were a few weak asphyctic muscle twitchings However, in dogs injected intravenously with 4 mgm of β erythroidine, violent struggling was frequently seen, when the breathing became irregular

Chronic toxicity   β 1 rythroidine was fed daily to three groups of 10 rats each in amounts of 10, 50 and 200 mgm per kgm respectively No untoward effects were produced by the feeding of 10 or 50 mgm per kgm over a period of nine months A daily dose of 200 mgm per kgm corresponding to about 40 per cent of the LD 50 (table 1) was well tolerated for two months After this period the rats lost gradually in weight, became emaciated and died On gross ex amination, no significant pathologic changes were observed in the organs

A group of five cats received 10 mgm /kgm of β erythroidine (almost one half of the lethal dose table 1) daily by subcutaneous injection for a period of three months During this period the weight of the animals remained stationary and no toxic effects became manifest

Curarizing effect   A *In frogs* The action of the Erythrina alkaloids on skeletal muscle was studied in frogs maintained at a constant temperature of 20°C Flaccid paralysis and cessation of respiratory movements were obtained with the intralymphatic injection of 3 mgm of β erythroidine per kgm This dose produced a complete paralysis within 30 minutes Larger amounts paralyzed the frog within periods as short as 5 minutes Faradization of the sciatic nerve failed to elicit muscle contractions where a stimulation of the muscle itself was always followed by contraction The threshold for direct muscle stimulation remained unchanged during the paralytic stage All substances listed in table 2 produced typical curare like paralysis in frogs

The potency of the various compounds was determined by the minimum dose producing complete curarization The summary in table 2 shows that the

dihydro compound is six times as potent as β-erythroidine itself. β-tetrahydro-β-erythroidine is as effective as dihydro-β-erythroidine, whereas α-tetrahydro-β-erythroidine possesses only a weak curarizing action. Alkaline hydrolysis of β-erythroidine reduced its efficacy considerably, as indicated by the threshold dose of 75 mgm. for sodium β-erythroidinate. On the other hand, hydrolysis of the dihydro compound leading to sodium dihydro-β-erythroidinate did not decrease appreciably its potency. The surprisingly low potency of β-erythroidine methiodide, a quaternary base, is of considerable interest.

TABLE 2

Potency of erythrina alkaloids in frogs

	MINIMUM CURARIZING DOSE BY INTRALYMPHATIC INJECTION
	mgm. per kgm.
β-erythroidine hydrochloride	3
Dihydro-β-erythroidine hydrochloride	0.5
α-tetrahydro-β-erythroidine hydrobromide	200 0
β-tetrahydro-β-erythroidine hydrobromide	0.5
Sodium-β-erythroidinate	75 0
Sodium dihydro-β-erythroidinate	0.6
β-erythroidine methiodide	200 0

TABLE 3

Duration of paralysis in frogs, expressed by the time interval between injection and complete recovery for the average of 10 frogs on each dose level

β-erythroidine		Sodium-β-erythroidine		Dihydro-β-erythroidine	
Mgm./kgm.	Duration	Mgm./kgm.	Duration	Mgm./kgm.	Duration
3	2.5 hrs.	75	30 hrs.	0 5	4 hrs.
6	5 hrs.	150	Died	1 0	8 hrs.
10	10 hrs.			1 5	12 hrs.
30	24 hrs.			5 0	24 hrs.
120	3 days			20 0	3 5 days
240	4 days			30 0	Died
300	Died				

A comparison of the curarizing potency of these alkaloids (table 2) and their toxicity (table 1) reveals an excellent agreement: the order of toxicity in warm blooded animals parallels closely that of the curarizing potency in frogs.

Threshold doses of β-erythroidine produced a very short lasting effect, the stage of complete paralysis was attained within 30 minutes and lasted for about half an hour. Thereafter the frogs recovered rapidly, regaining their previous motility within two to three hours after the injection. Increase of the dose of β-erythroidine increased the duration of the paralysis as shown in table 3. The effect of dihydro-β-erythroidine was of somewhat longer duration than that of β-erythroidine; full recovery from the effects of the minimum paralyzing dose

of this substance required four hours as compared to 2 5 hours following the threshold dose of β erythroidine   The smallest effective dose of sodium β erythroidinate caused paralysis lasting for 30 hours

Frogs tolerated amounts as large as 80 times the threshold dose of β erythroidine   They fully recovered from complete paralysis lasting for three days Still larger doses, representing a hundred fold of the threshold dose, produced paralysis lasting for five days from which the frogs did not recover   Likewise, increase in the dose of dihydro β erythroidine caused a prolongation of the paralysis, the lethal dose being 60 times greater than the paralyzing dose   The effect of sodium β erythroidinate, however, became irreversible when the minimum paralyzing dose was doubled

Carbaminoyl choline in small amounts which have no effect on the motility of frogs enhances and prolongs the effect of β erythroidine   Doses of 2 mgm per kgm of β erythroidine insufficient to produce complete paralysis were fully effective in paralyzing frogs which had received carbaminoyl choline   The duration of the paralysis obtained with 3 or 6 mgm per kgm of β erythroidine (table 3) was prolonged to 24 hours by the intralymphatic injection of 0 2 to 0 5 micrograms of carbaminoyl choline   The combination of larger amounts of carbaminoyl choline (2 to 20 micrograms per frog) and β erythroidine (3 and 6 mgm per kgm) produced death within 18 hours, although the amount of each substance given separately was not fatal

B *In cats* The nerve muscle action of β erythroidine was studied in cats anesthetized with urethane (0 8 gram per kgm) and chloralose (0 6 gram per kgm)   The leg was fixed by drills into the tibia   The sciatic nerve was severed and shielded platinum electrodes were used for the stimulation of its peripheral end   Shielded platinum on 2 wick electrodes applied to the popliteal nerve served for the recording of the action potential of the nerve whereasaction potentials of the small muscles of the toes were obtained through bipolar (Adrian Bronk) electrodes   The distance between the recording electrodes of the nerve and the muscle was between 8 and 10 cm

Sharp spiked monophasic stimuli of short duration were produced by a modified relaxation oscillator   The stimulator could be adjusted to deliver either a single discharge or a volley   The frequency could be varied from 1 to 250 per second   The recording electrodes were connected to a resistance capacity coupled amplifier, the output of which was fed to a 5 inch cathoderay tube Photographs were taken from the cathode ray oscillagraph with a 5 by 7 inch camera the shutter of which was synchronized with the stimulus and the single sweep of the electron beam   When the responses of both nerve and muscle were recorded on one plate, the exposures followed each other within 30 seconds

At the beginning of each experiment, nerve and muscle action potentials obtained by maximal stimulation with single discharges were recorded (fig 1,A) Immediately following intravenous injections of 0 5 to 1 0 mgm per kgm of β erythroidine a marked diminution in the amplitude of the action potential of the muscle was observed on stimulation at a rate of 1 per second, whereas the action potential of the nerve remained unaltered   The decrease in the amplitude

of the action potential of the muscle lasted for about five minutes. After 30 minutes the response to stimulation at low frequencies had returned to normal. Injection of 1 to 1.5 mgm. per kgm. abolished the action potentials of the toe muscles completely within 1 to 2 minutes (fig. 1,B). Increase of the stimulus, which is responsible for the increased amplitude of the action potential of the nerve in Fig. 1B, failed to elicit any response from the muscle. The transmission from nerve to muscle was completely blocked for about 10 to 15 minutes.
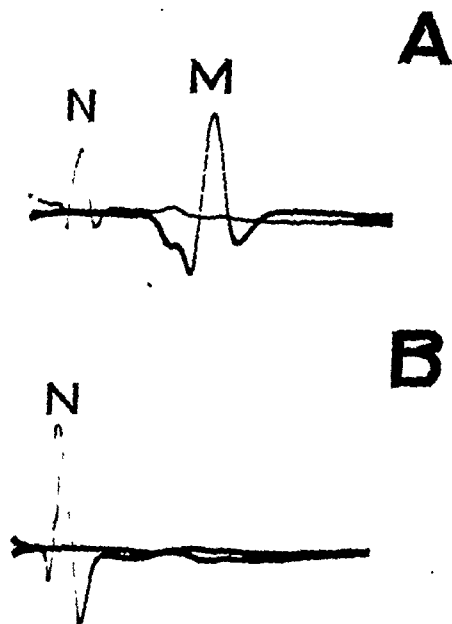
FIG. 1. EFFECT OF β-ERYTHROIDINE ON ACTION POTENTIALS OF POPLITEAL NERVE (N) AND TOE MUSCLE (M) FOLLOWING STIMULATION OF SCIATIC NERVE BY SINGLE DISCHARGE

Cat, 3 kgm., urethane-chloralose anesthesia. Time 10 m.sec. A. Before β-erythroidine. B. 5 minutes after intravenous injection of 1.5 mgm./kgm. β-erythroidine. Stimulus was increased.

Thereafter, stimulation was followed by slowly increasing responses of the muscle; after 30 to 60 minutes the action potential of the muscle had reached its original magnitude. Spontaneous breathing was maintained at a regular rate by the diaphragm if the doses of β-erythroidine did not exceed 1.5 mgm. per kgm. Larger doses of β-erythroidine administered under artificial respiration increased the duration of the paralysis of the muscle. Prostigmine in doses of 0.1 to 0.3 mgm. abolished completely the effect of small doses of β-erythroidine within 2 to 5 minutes and accelerated markedly the recovery from larger doses.

When prostigmine was given prior to the injection of β erythroidine the dose of the latter had to be increased from 1 0 or 1 5 mgm per kgm to at least 2 mgm per kgm in order to obtain complete block of the transmission from nerve to muscle

At stages of incomplete paralysis—either following an insufficient amount of β erythroidine or during recovery from a fully effective dose—indirect stimulation with volleys of 12 discharges caused a rapid decrease in the amplitude of the muscle potentials   The first muscle spikes obtained during repetitive stimulation were of the same magnitude as those following single discharges but the following spikes gradually decreased   The decrease in the amplitude depended on the frequency of the discharges   with high frequencies (60 and 120 per second) it was much more marked than with lower frequencies (10 and 30 per second)

FIG 2 EFFECT OF β ERYTHROIDINE UPON HEART RATE AND BLOOD PRESSURE
Dog
Upper
Hg T

EFFECTS ON CIRCULATION AND SMOOTH MUSCLE   Intravenous injections of both β erythroidine and dihydro β erythroidine caused a transient fall in blood pressure in rabbits cats and dogs anesthetized with sodium pentobarbital (fig 2)   Doses effective in depressing the blood pressure were below those causing paralysis of the skeletal muscle   Large doses administered under artificial respiration did not depress the blood pressure to an extent appreciably greater than smaller doses

In 7 dogs, β erythroidine and dihydro β erythroidine caused a marked slowing of the heart beat   In these experiments (fig 2) 2 mgm of β erythroidine per kgm , a dose insufficient to paralyze the skeletal muscle produced a decrease in the heart rate from 192 to 168 beats per minute   Larger doses produced a proportionally greater decrease in the heart rate   Following paralyzing doses the heart frequency often reached values as low as 50 or 60 per minute and the asphyxia due to the paralysis of the diaphragm failed to cause an acceleration of the heart rate   When the respiration had ceased, the heart was still beating

slowly and regularly. These alkaloids did not affect the normal sinus rhythm of the heart. The electrocardiogram showed no significant changes except for an increase in the atrio-ventricular conduction time.

In cats, a decrease in the heart frequency was not observed. In contrast to the findings on dogs, the heart rate of cats became accelerated when the injection of β-erythroidine caused paralysis of the diaphragm. Only occasionally was a decrease in frequency observed when very large doses of β-erythroidine were given under artificial respiration.

Vagotomy in dogs anesthetized with sodium pentobarbital did not prevent the decrease in the heart rate caused by β-erythroidine, or dihydro-β-erythroidine; atropine given in doses of 10 mgm. intravenously failed likewise to abolish the effect of β-erythroidine upon the heart rate (fig. 2). Hence, it appears unlikely that the decrease in the heart rate is of vagal origin. However, the administration of atropine prevented the bradycardia in non-anesthetized dogs, confirming the findings of Lehman, Chase and Yonkman (13).

On the smooth muscles of the isolated intestine of the rabbit, the *Erythrina* alkaloids were without effect. Concentrations up to 1:20,000 did not alter the intestinal tone nor the rhythmic contractions.

ANTAGONISM WITH PROSTIGMINE. The effect of physostigmine salicylate and of prostigmine upon the action of *Erythrina* alkaloids was studied in frogs, mice, rabbits, cats and dogs. Parenteral injections of prostigmine promptly abolished the toxic effects of β-erythroidine and of dihydro-β-erythroidine in all species. Physostigmine, however, had little effect in counteracting the action of β-erythroidine in frogs. Intralymphatic injection of 0.2 mgm./kgm. failed to influence the paralysis caused by 6 mgm./kgm. β-erythroidine, whereas larger doses (1.0 and 1.2 mgm./kgm.) slightly accelerated the recovery from the paralysis. On the other hand, 0.1 mgm./kgm. of prostigmine rapidly abolished the effects of β-erythroidine; within less than one hour the frogs had completely recovered. In cats and dogs, whose respiration had become irregular or had ceased entirely following lethal doses of β-erythroidine, intravenous injections of prostigmine or physostigmine immediately restored the respiration to a regular rate and within 2 or 3 minutes the animals regained muscular strength and were able to move about. In a total of 50 experiments with or without anesthesia on rabbits, cats and dogs, prostigmine regularly abolished the effects of β-erythroidine or its derivatives, provided it was administered before the terminal collapse of the circulation. The antagonistic effect of prostigmine upon the paralysis of the respiration following β-erythroidine is illustrated in an experiment on an anesthetized cat in figure 3. In this experiment the injection of a fatal dose of β-erythroidine (6 mgm./kgm.) was followed by a depression of both blood pressure and respiration. The respiration became shallow, slower and irregular, and all respiratory movements ceased 2 minutes after the injection whereas, the blood pressure regained its original level. The injection of 0.1 mgm. prostigmine caused prompt resumption of the respiration which rapidly returned to the regular rhythm at its previous rate. Larger doses of prostigmine were often followed by generalized muscular fibrillation, salivation and defecation.

Premedication with prostigmine prevented the toxic effects of the *Erythrina* alkaloids and rendered otherwise lethal doses ineffective The quantitative relation of the antagonism between prostigmine and both β erythroidine and
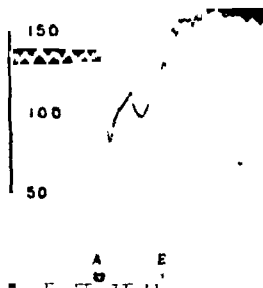
FIG 3 ANTAGONISTIC EFFECT OF PROSTIGMINE ON RESPIRATORY PARALYSIS CAUSED BY
β ERYTHROIDINE

Cat 2 5 kgm   sodium pentobarbital 35 mgm /kgm intraperitoneally   Upper tracing Respiration   Lower tracing Carotid blood pressure   Time 30 seconds   A β erythroidine 6 mgm /kgm   B Prostigmine 0 1 mgm

dihydro β-erythroidine was studied on mice The toxicity of the *Erythrina* alkaloids was determined in two groups of animals which received a subcutaneous injection of 0 3 mgm per kgm of prostigmine prior to the injection of various doses of β erythroidine and dihydro β erythroidine respectively In the same manner, the toxicity of prostigmine was determined in mice which had received

a subcutaneous injection of 50 mgm. β-erythroidine or of 10 mgm. dihydro-β erythroidine respectively. The results obtained on a total of 250 mice and expressed by curves calculated from the deduced mortality (14) of the various dose levels are presented in figure 4.

As evidenced by figure 4, premedication with prostigmine rendered the animals more resistant to the *Erythrina* alkaloids; the L.D. 50 of either β-erythroidine or dihydro-β-erythroidine increased by about $2\frac{1}{2}$ times. Likewise, premedication with *Erythrina* alkaloids decreased the toxicity of prostigmine to almost the same extent, as shown by a comparison of the L.D. 50 of 0.47 mgm. per kgm. to that of 1.03 and 1.25 in mice which were treated with β-erythroidine of dihydro-β-erythroidine respectively. All animals in these experiments receiving lethal doses of both prostigmine and the *Erythrina* alkaloids succumbed
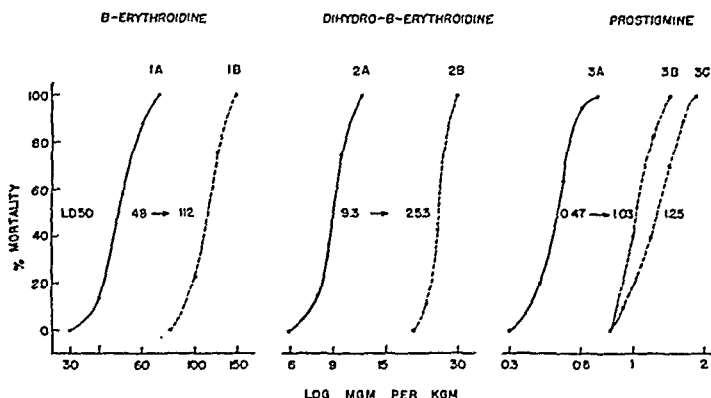

FIG. 4. ANTAGONISM BETWEEN ERYTHRINA ALKALOIDS AND PROSTIGMINE

Influence of premedication upon toxicity in mice: 1A. Toxicity of β-erythroidine. 1B. Same following 0.3 mgm./kgm. prostigmine. 2A. Toxicity of dihydro-β-erythroidine. 2B. Same following 0.3 mgm./kgm. prostigmine. 3A. Toxicity of prostigmine. 3B. Same following 50 mgm./kgm. β-erythroidine. 3C. Same following 10 mgm./kgm. dihydro-β-erythroidine.

to respiratory failure; the survival time of these animals was not significantly increased by the administration of the antagonist.

Anti-convulsant action. The effect of β-erythroidine upon metrazol convulsions was studied in four dogs. Intravenous injections of 25 mgm. per kgm. of metrazol produced severe convulsive seizures lasting from one to four minutes. Two days later, the same dose of metrazol was given immediately after an intravenous injection of 4 mgm. per kgm. of β-erythroidine. The convulsions were markedly mitigated in two dogs and almost entirely suppressed in the two other animals. All dogs recovered rapidly within fifteen minutes. When the dose of β-erythroidine was increased to 6 mgm. per kgm., the subsequent administration of metrazol failed to produce any seizures, but respiratory failure developed in three dogs within 5 to 8 minutes after the injections of β-erythroidine and metrazol. In these animals, intravenous injections of 0.5 mgm. prostigmine

caused a prompt increase in the rate and depth of the respiration   Within three minutes the regularity of the respiration was restored and all animals made a quick recovery

Discussion   The experiments herein reported demonstrate that β Ery throidine and the substances derived from it possess true curare action directed specifically against the myo neural junction   The sequence in which the various skeletal muscles become paralyzed appears to be the same as that observed with curare (15)   Paralysis of the skeletal muscle precedes that of the diaphragm and death due to asphyxia ensues in homothermic animals   The margin be tween the dose of the alkaloid causing paralysis of the skeletal muscle and that paralyzing the diaphragm, although small, is more distinct than with curare   Following the administration of a suitable dose of dihydro β erythroidine cats may remain in a stage of almost complete paralysis for two hours and recover rapidly thereafter without need for artificial respiration, breathing sufficient to prevent asphyxia is maintained by the activity of the diaphragm throughout the paralytic stage

On the other hand, using curare preparations of various origin we have been unable to paralyze rabbits or cats to such a degree that the diaphragm was still functioning while the skeletal muscles were completely paralyzed

Block of the transmission of nerve impulses to the skeletal muscle is the paramount action of the *Erythrina* alkaloids   A multiple of the paralyzing dose prolongs the duration of paralysis in frogs, and a one hundred fold of the ef fective dose of β-erythroidine is required to produce death in frogs   Cats main tained under anesthesia and artificial respiration tolerated ten fold the paralyzing dose   The smooth muscles of the intestinal organs are not affected by β erythroidine, nor did we, in agreement with Rosen et al (16) and Brown et al (17), encounter salivation, vomiting defecation and gastro intestinal distress, as seen in dogs by Lehman, Chase and Yonkman (13)

The curarizing potency of the *Erythrina* alkaloids appears to be of the same order as that of purified preparations of curare   Curarine, the most potent alkaloid isolated by King (18) from Strychnos toxifera paralyzed frogs at a dose of 0 5 mgm per kgm (19), in our experiments, dihydro β erythroidine was effective at the same dosage

β Erythroidine and its derivatives differ, however, from curare with regard to absorption, duration of action and excretion   β Erythroidine and dihydro β erythroidine are rapidly absorbed from the gastro intestinal tract, in some species, they are almost as effective by oral administration as by subcutaneous injection   The rapid onset of paralysis following oral administration in less than 15 minutes, reported also by Lehman (17) in experiments on dogs, makes it likely that the *Erythrina* alkaloids are largely absorbed from the stomach   The duration of the paralysis following these alkaloids especially β-erythroidine is remarkably short when compared to curare   The action of dihydro β erythroidine was in all species longer lasting than that of β-erythroidine   In experiments with prolonged daily administration of β erythroidine no indica tion of cumulative effects of this substance was obtained   The alkaloid appears

to be rapidly metabolized after entering the circulation. Urine collected from frogs paralyzed by large doses of the alkaloid failed to have any paralyzing effect upon other frogs, whereas curare is known to be excreted unaltered in the urine. Thus, it is unlikely that β-erythroidine is excreted by the kidney in its pharmacologically active form.

In rabbits, dogs and cats, intravenous injections of β-erythroidine as well as of dihydro-β-erythroidine produce a transient fall in blood pressure. Bradycardia produced by these alkaloids in dogs anesthetized with sodium pentobarbital, and only rarely and to a much lesser extent in cats, is apparently not due to mediation of the vagal nerve, since neither vagotomy nor atropinization prevented the cardiac slowing. These experiments suggest that the bradycardia is due to a direct action upon the heart. In non-anesthetized dogs Lehman et al. (13), reported a prevention of the bradycardia by atropine. We have confirmed his work on non-anesthetized animals. The atrio-ventricular conduction time is prolonged. Evidence of an increased conduction time in man during β-erythroidine administration has been reported by Williams (20).

No evidence for effects upon the central nervous system has been obtained in our experiments. In particular, hypnotic or sedative effects as reported in man (21), (22) have not been observed in animals.

The anticonvulsant effect of *Erythrina* alkaloids was first demonstrated with crude extracts from seeds of *Erythrina americana* Mill. by Lehman (6) in controlling the convusions of strychnine, camphor, cocaine and picrotoxin in animals. Rosen and co-workers (16), obtained mitigation and suppression of metrazol seizures in dogs by β-erythroidine. This observation confirmed by Lehman (13) and by our experiments has led to the use of β-erythroidine as an adjunct in the metrazol therapy of psychoses (Rosen, Cameron and Ziegler (23) and others).

Physostigmine and prostigmine both known to antagonize the effect of curare also exert an antagonistic effect upon *Erythrina* alkaloids. This antagonism is mutual, prostigmine abolishing the effects of β-erythroidine as well as this alkaloid preventing the action of prostigmine. Prostigmine appears to be a reliable and prompt acting antidote which takes its place with artificial respiration in cases of overdoses of *Erythrina* alkaloids.

The antagonism with prostigmine led to speculation whether β-erythroidine influences the choline esterase activity. Through the courtesy of Dr. D. Glick the effect of β-erythroidine was studied in vitro. The presence of 50 mgm percent of the drug had no demonstrable influence on the cholinesterase activity of a 0.5 percent solution of horse serum. Later, Harris and Harris (24) failed to obtain any effect of this alkaloid upon choline esterase activity in human serum. Hence, it appears unlikely that the effect of β-erythroidine and its antagonistic effect upon prostigmine is due to a direct action upon choline esterase.

Compared to prostigmine, physostigmine is much less effective in antagonizing the action of β-erythroidine in frogs whereas in homothermic animals both substances exert about the same antagonistic effect upon the action of β-erythroidine. A similar difference between physostigmine and prostigmine in their

effectiveness in reversing the action of curare in frogs had been observed by Jacobsohn and Kahlson (25) Carbaminoyl choline known to enhance the effect of curare (25) also enhances the action of β erythroidine in frogs

Conversion of β erythroidine into the quaternary metho salt (β erythroidine methiodide) decreases its curarizing action to about one hundredth of the activity of the tertiary base Since the discovery of the curariform properties of quaternary bases by Crum Brown and Fraser and their observations that through conversion into methiodides or methosulfates many alkaloids with specific action upon the central nervous system acquire curariform properties, it has been established that the curariform action of a substance generally is linked to the presence of a quaternary nitrogen configuration in the molecule To these observations Boehm's studies on curare alkaloids (26) have added a particularly striking example From various curares and from the bark of Strychnos toxifera Boehm isolated "curin", tertiary ammonium bases with little or no neuro muscular action, conversion into their methiodides increased the potency of these substances to such a degree that it equalled the potency of the quaternary base—' curarin'—isolated simultaneously from the same material β Erythroidine, therefore, constitutes a notable exception to the generalization of the classical findings of Crum Brown and Fraser on the relationship between chemical structure and curare activity

SUMMARY

1 β Erythroidine and substances derived from it (dihydro β-erythroidine, α tetrahydro β-erythroidine, β tetrahydro β-erythroidine, sodium β erythroidine, sodium dihydro β erythroidine, and β erythroidine methiodide) possess typical curare like action

2 Compared with curare, the paralysis caused by these alkaloids, especially β-erythroidine, is of short duration

3 Dihydro β-erythroidine is the most potent of these alkaloids, it equals cur arine in potency

4 β Erythroidine and dihydro β-erythroidine are effective by oral adminis tration   The toxicity has been determined in mice, rats, rabbits and cats fol lowing subcutaneous and oral administration

5 Death in mammals is caused by paralysis of the diaphragm, the heart continues to beat in regular rhythm for several minutes

6 Intravenous injections of β-erythroidine or dihydro β erythroidine cause a transient fall in blood pressure   In dogs they decrease markedly the heart rate and increase the atrio ventricular conduction time

7 Atropine prevents the bradycardia in non anesthetized dogs, but fails to influence the bradycardia in animals anesthetized with sodium pentobarbital

8 The smooth muscle is not affected by β erythroidine

9 Prostigmine is an effective antidote against β erythroidine and dihydro β erythroidine   The antagonism between prostigmine and the two *Erythrina* alkaloids is mutual

10 In contrast to the well known relation of tertiary and quaternary am-

monium groups in curare alkaloids to their neuro-muscular action, conversion of β-erythroidine into the quaternary metho salt (β-erythroidine methiodide) decreases the curarizing action to about one-hundredth of the tertiary base.

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PHARMACOLOGIC ACTION OF ERYTHRINA ALKALOIDS
II FREE, LIBERATED AND COMBINED ALKALOIDS

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In studies covering fifty-one out of the one hundred and five species of *Erythrina* known at present, it was found that the seeds of all species investigated possess curare like action when tested on frogs (1) The curarizing potency of the seeds, varying in wide limits among the different species but surprisingly uniform in samples of any one given species gathered from widely separated regions, appears to be a characteristic feature of the genus, *Erythrina* It has been shown by Folkers and his associates that the seeds of Erythrina contain a mixture of hitherto unknown alkaloids

Subsequent to the isolation of erythroidine from *Erythrina americana* Mill (2) other alkaloids have been isolated some of which, like β-erythroidine, are readily soluble in water as well as in chloroform Among these alkaloids, erythramine, erythraline and erythratine have been obtained in an established pure form Studies on the constitution of these substances (3) revealed that erythraline, $C_{18}H_{19}NO_3$, differs from erythramine, $C_{18}H_{21}NO_3$, by the presence of one additional ethylenic double bond, erythratine, $C_{18}H_{21}NO_4$, contains an additional non phenolic hydroxyl group All three alkaloids contain a tertiary nitrogen atom and react with methyl iodide to give the corresponding methiodides

The pure alkaloids produce the same curarizing effect as that observed with crude seed extracts However, the yield and potency of the alkaloids isolated from the chloroform fraction failed to account for the high curarizing activity of the seed extracts previously reported (1) For instance, seeds of *Erythrina glauca* Willd (sample Haigh 9170) contain only 0 4% or 4 mgm of these alkaloids per gram, an amount barely sufficient to paralyze 1 kgm of frog, whereas the extract of one gram of seed was found to paralyze about 33 3 kgm of frog From this and other experiments it became apparent that the activity of the seed extracts did not always represent a measure of the amount of alkaloids which could be isolated as the free[1] fraction This discrepancy between the potency of the seed extracts and the yield of free alkaloids suggested further chemical and pharmacological studies on the crude extracts After removal of the free alkaloidal fraction, aqueous extracts of the seeds of *Erythrina glauca* Willd and of other species were found to possess almost the same or only slightly less activity when retested on frogs It was therefore concluded that the seeds

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[1] This fraction has been designated as the free alkaloidal fraction to distinguish it from the fraction liberated by hydrolysis The latter has been designated as the liberated alkaloidal fraction and the combined alkaloidal fraction refers to this fraction before hydrolysis (5)

contain besides the free alkaloidal fraction other alkaloids of quite different solubility characteristics.

From the combined fraction Folkers and his associates have isolated by acid hydrolysis a new series of alkaloids of which erysopine, erysovine, erysodine and erysonine have been established in pure form (5, 6). The names of these liberated alkaloids were derived from the stem eryso- in order to distinguish them from the free alkaloids for which the stem erythr- was selected. Prior to hydrolysis, the liberated alkaloids exist in a combined form. Further chemical research showed the combined forms were alkaloidal esters of sulfoacetic acid (7). Thus, hydrolysis of two pure isolated combined alkaloids, erysothiopine and erysothiovine, gave erysopine and erysovine respectively and one molecule of sulfoacetic acid.

This paper is concerned with the pharmacologic action of various free, liberated and combined Erythrina alkaloids and some substances derived from these alkaloids. Their action has been compared to that of β-erythroidine (8).

The substances used in this study were obtained through the courtesy of Dr. Karl Folkers. The free alkaloids erythramine, erythraline and erythratine were available in the form of their hydrobromides and hydroiodides; dihydro-erythramine and dihydro-erythratine in the form of their hydrobromides. All substances are crystalline and readily soluble in water. The quaternary metho-salts of erythramine and erythratine were obtained through the reaction of the tertiary bases with methyl iodide.

The liberated alkaloids erysopine, erysovine, erysodine and erysonine were available as free bases, and were used in aqueous solutions in the form of their hydrochlorides, erysodine also in the form of its sodium salt. Of the hydrogenated derivatives, tetrahydro-erysopine and tetrahydro-erysodine were obtained as hydrobromides. The combined alkaloids were represented by the sodium salts of erysothiopine and erysothiovine.

Curarizing effect in frogs. The potency of the alkaloids was tested in frogs kept at a constant temperature of 20°C. All alkaloids, with the exception of tetrahydro-erysodine, were found to possess curarizing effects. Electric stimulation of the sciatic nerve failed to elicit any response from the gastrocnemius muscle, whereas direct stimulation of the muscle was effective. The threshold for the direct stimulation remained unchanged during the paralytic stage. The minimum doses of the various substances causing complete block of the neuromuscular transmission are summarized in table 1.

Erythramine and erythraline possessed the same potency (10 mgm. per kgm.) whereas erythratine was much less effective. Dihydro-erythramine and dihydro-erythratine were markedly less effective than the parent substances, and the conversion of either one of the three free alkaloids into their respective metho-salts decreased their curariform action. Among the liberated alkaloids erysovine was the most potent, closely followed by erysopine. Erysovine was equally effective in the form of the hydrochloride and as the sodium salt. Of the hydrogenated derivatives, tetrahydro-erysopine had only one-tenth of the activity of erysopine, and tetrahydro-erysodine failed to cause complete curarization when tested at 300 mgm. per kgm., thirty fold the effective dose of ery-

sodine The combined alkaloids erysothiopine and erysothiovine were equally effective, surpassing in potency all other substances

The average duration of the paralysis and its prolongation by increased doses of various alkaloids are summarized in table 2 The paralysis caused by the free alkaloids was short lasting, whereas the liberated and combined alkaloids had a prolonged effect Erysopine had the most persistent action of any of the alkaloids A threshold dose of this substance paralyzed the frogs for 24 hours,

TABLE 1

Potency of erythrina alkaloids in frogs

	MINIMUM CURARIZING DOSE BY INTRALYMPHATIC INJECTION
	mgm per kgm
Free alkaloids	
Erythramine HBr	10
Dihydro-erythramine HBr	300
Erythramine methiodide	40
Erythraline HBr	10
Erythraline methiodide	50
Erythratine HBr	75
Dihydro erythratine HBr	100
Erythratine methiodide	300
Liberated alkaloids	
Erysopine HCl	4
Tetrahydro erysopine HBr	40
Erysovine HCl	3
Sodium erysovinate	3
Erysodine HCl	10
Tetrahydro-erysodine HBr	>300
Erysonine HCl	100
Combined alkaloids	
Sodium erysothiopinate	1
Sodium erysothiovinate	1

ten fold this amount produced paralysis lasting for about 5 days from which some frogs failed to recover In the duration of its action, erysopine was distinctly different from erysovine The combined alkaloids, erysothiopine and erysothiovine however, had the same long lasting effect

Toxicity in mice The toxicity of some of the alkaloids was determined in mice following subcutaneous or oral administration, 10 animals being used for each dose level (table 3) All animals receiving lethal doses apparently died of respiratory failure due to paralysis of the diaphragm Death usually occurred within 1 to 3 hours The heart continued to beat for several minutes

after the respiration had ceased. Among the free alkaloids erythramine was less toxic than erythraline although both alkaloids were equally effective in frogs. Of the liberated alkaloids, erysopine was the most toxic. Erysothiopine, contrary to its effectiveness in frogs, was markedly less toxic than erysopine when tested on mice by subcutaneous injection. The oral toxicity was determined for erythraline, erysodine and erysopine. In all instances, the toxic doses were from 10% to 50% larger than those established by subcutaneous injection.

TABLE 2

Duration of paralysis in frogs, expressed by the time interval between injection and complete recovery for the average of 10 frogs on each dose level

ERYTHRAMINE ERYTHRALINE		ERYSOVINE		ERYSOPINE		ERYSODINE		ERYSOTHIOPINE ERYSOTHIOVINE	
Mgm / kgm	Duration	Mgm / kgm.	Duration	Mgm / kgm	Duration	Mgm / kgm.	Duration	Mgm / kgm	Duration
10	3 hrs.	3	6 hrs.	4	24 hrs.	10	10 hrs.	1	24 hrs.
100	2 days	6	10 hrs.	8	1.5 days	20	24 hrs.	3	30 hrs.
200	3 days	10	15 hrs.	12	2 5 days	40	2 days	10	2 days
400	Died	20	24 hrs.	20	3.5 days	100	Died	50	Died
		30	2 days	40	5 days				
		100	Died	100	40% Died Died				

TABLE 3

Toxicity of various erythrina alkaloids in mice

	LD 50 (MGM. PER KGM)	
	Subcut.	Oral
Free alkaloids		
Erythramine HBr	104	
Erythraline HBr	72	80
Liberated alkaloids		
Erysopine HCl	14.8	18
Erysodine HCl	100	155
Combined alkaloids		
Sodium erysothiopinate	76	

Effect on circulation. The effect of intravenous injections of various alkaloids was studied in rabbits, cats and dogs anesthetized with sodium-pentobarbital. The free alkaloids, erythramine and erythraline showed no significant difference in their action upon the blood pressure and the heart rate; the intravenous injection of 3 to 5 mgm. per kgm. of either one of these alkaloids was fatal in cats. Both alkaloids caused a sharp fall in blood pressure returning to its original level within 3 to 15 minutes depending on the amount injected (0.25 to 1.5 mgm. per kgm.). Thus, the effect of these alkaloids on the blood pressure was of

the same short duration as observed with β-erythroidine (8) but the fall in
blood pressure was more marked than that following β-erythroidine Further-
more, in contrast to β-erythroidine, erythramine and erythraline significantly
decreased the heart rate in cats, when doses smaller than the paralyzing dose
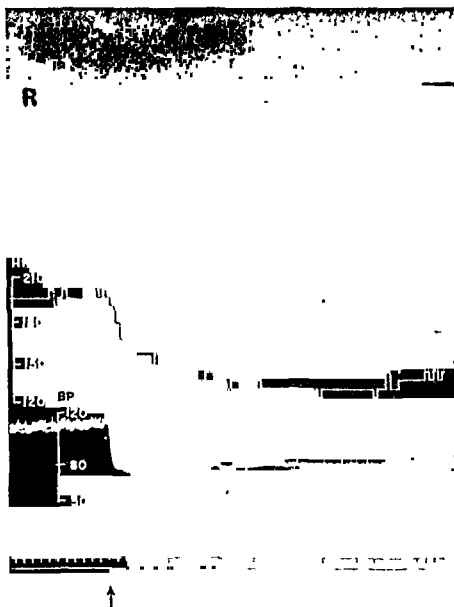
FIG 1 EFFECT OF ERYSOPINE ON HEART RATE AND BLOOD PRESSURE

were injected   The slowing of the heart rate was transient, its duration paral-
leling that of the depression of the blood pressure   Intravenous injections of
atropine (2 mgm. per kgm) failed to prevent either the decrease in heart rate
or the fall in blood-pressure

The liberated alkaloids, erysopine and erysovine caused decrease in the
blood pressure and slowing of the heart rate which was more pronounced than
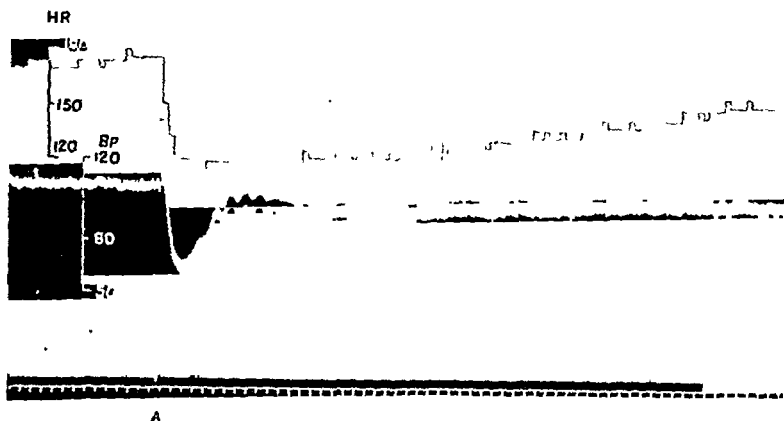
FIG. 2A. EFFECT OF ERYSOVINE ON HEART RATE AND BLOOD PRESSURE

Dog, 6 kgm., anesthetized with 36 mgm. per kgm. sodium pentobarbital intraperitoneally. HR: Heart rate, beats per minute. BP: carotid blood pressure, mm Hg. Time in 30 seconds. The animal was given a total of 4 mgm per kgm atropine sulfate intravenously. A, erysovine, 0.5 mgm per kgm.

FIG. 2B. EFFECT OF ERYSOTHIOVINE ON HEART AND BLOOD PRESSURE

Same experiment as in Fig. 2A.   B, erysothiovine, 0.25 mgm per kgm.

than that observed with any of the free alkaloids   Also, the duration of these effects on the circulation was prolonged   Erysovine appeared to be slightly more effective than erysopine   Erysopine, howe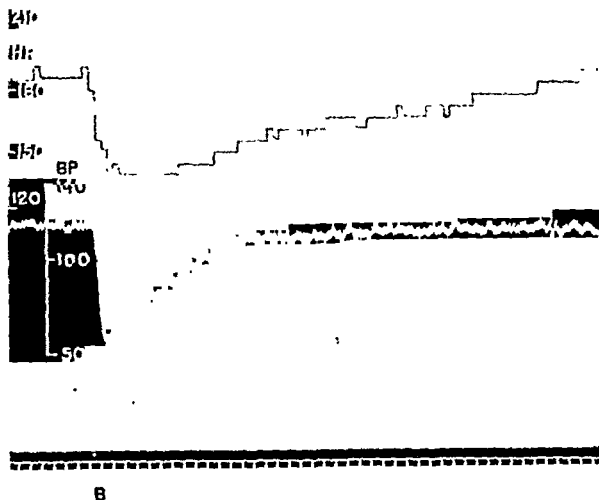ver, surpassed erysovine in the duration of its depressing effects upon both blood pressure and heart rate   Figure 1 illustrates the effect of erysopine on an anesthetized cat, the dose of 1 mgm per kgm did not influence the respiration, but slowed the heart rate from 200 to 130 beats per minute and lowered the blood pressure from 110 to 70 mm Hg   Blood pressure and heart rate gradually regained their original levels within 45 minutes   In dogs, depression of the blood pressure and of the heart rate were observed with doses of 0 2 mgm per kgm of erysovine, representing less than 10% of the paralyzing dose   The effect of 0 5 mgm per kgm erysovine upon the heart rate and blood pressure of a dog is illustrated in figure 2A   The slowing of the heart rate (from 175 to 120 beats per min) persisted much longer than the depression of the blood pressure   It subsided gradually and 45 minutes after the injection, the heart rate reached its previous frequency

The effects on blood pressure and heart rate in these experiments were not influenced by atropine   In dogs anesthetized with sodium pentobarbital atropine often failed to increase the heart rate significantly   In order to be assured that sufficient atropine had been given to paralyze the vagal endings, either the vagal nerves were stimulated or the effect of carbaminoyl choline was tested before and after atropine   Dogs anesthetized with sodium pentobarbital required comparatively large amounts of atropine for the abolishment of vagal responses   In atropinized dogs, erysovine (fig 2A) and erysothiovine (fig 2B) still produced a marked decrease in both heart rate and blood pressure   These effects were not significantly different from those obtained without atropinization

Erysothiovine (fig 2B) and erysothiopine caused a depression of both the heart rate and the blood pressure comparable to that obtained with erysovine and erysopine   The effective doses of the combined alkaloids, however, were smaller than those of the liberated alkaloids

DISCUSSION   All Erythrina alkaloids investigated possess true curare action, although the potency varies greatly among the different alkaloids   They also differ markedly in the duration of their action   Differences in potency and duration of effects between β-erythroidine and some substances derived from it particularly dihydro β-erythroidine, have already been reported (8)   A comparison of the efficacy of the free, liberated and combined alkaloids and the duration of their action in frogs is presented in figure 3

The free alkaloids, β-erythroidine, erythramine and erythraline share a characteristic feature in that their action is remarkably short   The smallest effective doses ranging from 3 to 100 mgm per kgm are similar in both the free and liberated alkaloids, but the action of the liberated alkaloids is more persistent   Erysovine, for instance, causes paralysis lasting more than twice as long as that following β-erythroidine, although the smallest effective doses of both substances are the same   On the other hand   the combined alkaloids erysothiopine and erysothiovine are effective in much smaller amounts than the

corresponding liberated alkaloids. The duration of their effect is either equal to, (erysothiopine) or more prolonged (erysothiovine) than, that of the liberated alkaloids.

The duration of the paralysis caused by these alkaloids can be prolonged by increasing the dose. Of the free alkaloids, erythramine, erythraline and β-erythroidine, frogs tolerate amounts from 20 to 80 times greater than the smallest effective dose, recovering completely after 3 to 5 days. The effect of the liberated alkaloids, erysodine and erysopine, however, becomes irreversible when 10 times the threshold dose is given. The difference in the spread from the minimum effective to the lethal dose between the free and liberated alkaloids apparently indicates that the short acting free alkaloids are metabolized at a faster rate than the liberated alkaloids.
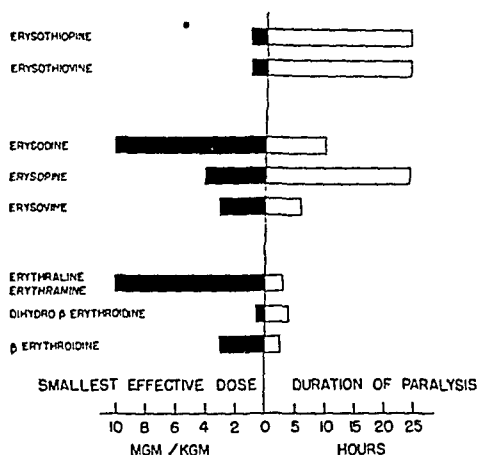

FIG. 3. COMPARISON BETWEEN THE EFFICACY AND THE DURATION OF THE ACTION OF ERYTHRINA ALKALOIDS IN FROGS

In general, the order of toxicity of the alkaloids in mice corresponds to that of their potency in frogs. A notable exception was found in erysothiopine which in the form of the sodium salt[2] was much less effective in mice than was anticipated from its high potency in frogs. On intravenous injection in cats, however, erysothiopine was more effective than erysopine; paralysis of the diaphragm and death were obtained with 0.8 mgm. per kgm. erysothiopine as compared to 2 mgm. per kgm. erysopine.

The circulatory effects of the various alkaloids reported in this study are more pronounced than those obtained with β-erythroidine (8). This is especially true for the liberated and combined alkaloids which lower the heart rate and depress the blood pressure in quantities which are only fractions of their paralyz-

[2] The sodium salts of the alkaloids were less stable on standing in aqueous solutions (pII 10-14) than solutions of the hydrochlorides which showed unaltered activity when retested after 3 months.

ing doses. Atropine given in doses sufficient to paralyze the vagal ending fails to prevent the decrease in heart rate in animals anesthetized with sodium pentobarbital. Hence, it is concluded that the bradycardia observed is due to a direct action of these alkaloids upon the heart.

Hydrogenated derivatives of free alkaloids, (dihydro-erythramine and dihydro-erythratine) and of liberated alkaloids, (tetrahydro-erysopine and tetrahydro-erysodine) were considerably less active on frogs than their parent alkaloids. This is in contrast to the findings with the hydrogenated derivatives of β-erythroidine; dihydro-β-erythroidine was about 6 times more active in frogs than β-erythroidine.

Conversion of erythramine, erythraline and erythratine into their methiodides decreased their curarizing action to one-fourth or one-fifth. An even greater decrease in activity had been obtained by the conversion of β-erythroidine into β-erythroidine methiodide. Thus, the quaternary metho-salts of all free alkaloids were found to be less active than the tertiary bases.

SUMMARY

1. The following 9 alkaloids isolated from seeds of species of the genus *Erythrina*: erythramine, erythraline and erythratine (free alkaloids), erysopine, erysovine, erysodine and erysonine (liberated alkaloids), and erysothiopine and erysothiovine (combined alkaloids) produce typical curare-like action.

2. The potency of these alkaloids and of some derivatives has been determined on frogs; the greatest activity was found with the combined alkaloids.

3. The combined and the liberated alkaloids have a more persistent action than the free alkaloids.

4. The order of toxicity of various alkaloids in mice following oral and subcutaneous administration followed, in general, that of their potency in frogs.

5. Intravenous injections especially of the liberated and combined alkaloids depressed the blood pressure and lowered the heart rate to a greater extent than did β-erythroidine.

6. Atropine failed to influence the bradycardia in cats and dogs anesthetized with sodium pentobarbital.

7. In contrast to the findings with β-erythroidine and dihydro-β-erythroidine, hydrogenated derivatives of erythramine, erythratine, erysopine and erysodine are less active than the parent alkaloids.

8. The quaternary metho-salts of erythramine, erythraline and erythratine are, like that of β-erythroidine, less active than the tertiary bases.

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A PHARMACOLOGICAL STUDY OF AN EXTRACT OF ERYTHRINA CRISTA GALLI (CEIBO)

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The curarizing effect of extracts of Erythrina crista galli (ceibo) was described toward the end of the last century (for old references see Cicardo and Hug (1)), but its mode of action has not been thoroughly worked out. The aim of the present work was to study the curarizing action of the alkaloids of Erythrina and to see if they have any other pharmacological effects of interest.

METHODS. All the experiments were done on cats weighing 2½ to 3 kgm., anesthetized with dial (Ciba, 0.75 cc. per kgm. injected intraperitoneally). When in rare cases this anesthesia was insufficient, it was supplemented by urethane (25%) injected intravenously.

A. *Skeletal muscle.* M. quadriceps was used. The leg was fixed by means of drills in the two ends of the femur. Electrical stimulation of the crural nerve was affected by condenser discharges timed by an oscillator. Injections, both of acetylcholine, used as a stimulant of denervated muscles, and of the Erythrina, were made into the femoral artery close to the muscle so that the latter would receive most of the injected drug.

B. *Nictitating membrane.* After enucleation of the eye the contractions of the nictitating membrane were recorded on stimulation of the pre- and postganglionic nerves. Acetylcholine was injected into the carotid artery in doses of 30 to 50 micrograms.

C. *Pupil.* The pre- and postganglionic parasympathetic nerves as well as the postganglionic sympathetic nerves were stimulated. For stimulation of the preganglionic parasympathetic fibers the electrodes were placed on the third nerve where it passes through the bony canal, leaving the cranial cavity and entering the orbital cavity. Here the nerve is isolated but very fragile because it has lost its sheath. For stimulation of the postganglionic parasympathetic fibers the technique described by Luco and Salvestrini (2) was employed. Erythrina was injected into the carotid artery except in the experiments in which sympathetic and parasympathetic fibers were stimulated simultaneously. In these cases it was injected intravenously.

D. *Submaxillary gland.* The rate of salivary secretion was determined by recording, on a kymograph, the drops falling from a cannula placed in Wharton's duct. The chorda tympani was stimulated, and acetylcholine was injected intraarterially in doses of 10 to 20 micrograms.

In all the experiments the electrical stimuli were maximal. The stimuli were condenser discharges controlled either by electronic valves or by a Harvard inductorium whose primary coil was connected to the secondary of a step-down transformer delivering 4 volts at 50 cycles per second.

The following drugs were used: an alcoholic extract of Erythrina seeds (obtained from Prof. Hug, of the Instituto de Farmacología in Rosario, Argentina), diluted to 1/10, and an aqueous solution of the residual after distillation of the alcoholic extract, also diluted to 1/10; acetylcholine chloride (Merck); prostigmine (Roche).

RESULTS. A. *Skeletal muscle.* 1. *Indirect electrical stimulation:* Various doses of Erythrina injected during electrical stimulation at various frequencies caused in all cases a depression of the response (figs. 1, 2, and 3). This confirms the depressing effect of this drug on skeletal muscle described by Cicardo and Hug (1).

If a slow test frequency (6 to 25 per min.) was interrupted for 5 to 20 sec. by a tetanizing frequency during the depression produced by Erythrina, there occurred a post-tetanic decurarization similar to that observed by Boyd (3) with curare (fig. 3). The injection of various doses of prostigmine during the Erythrina depression also produced recovery (fig. 2), similar to what is seen in curare depression.
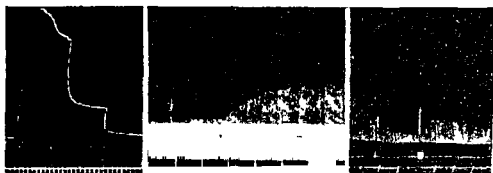

FIG 1   FIG. 2   FIG. 3

FIG 1 Effect of Erythrina on the contraction of M quadriceps stimulated indirectly

mark, tetanizing current at a frequency of 300 per sec for 20 sec Lower signal 1 min intervals
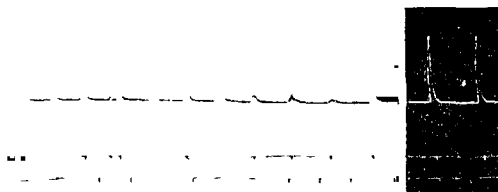
FIG 4 Stimulation of M quadriceps, denervated 8 days previously, by intrafemoral injections of acetylcholine in doses of 30 micrograms Upper signal each mark indicates the moment of injection of acetylcholine, the arrow indicates the injection of 0 20 cc of alcoholic extract of Erythrina Lower signal 1 min. intervals
Note at the right complete recovery after ½ hr

2. *Stimulation by acetylcholine·* The injection of Erythrina during stimulation by acetylcholine of muscles denervated 8 to 12 days previously produced a decrease of the response to acetylcholine (fig 4).

B. *Nictitating membrane.* 1. *Indirect electrical stimulation:* The administration of Erythrina during stimulation of the preganglionic fibers produced a depression of the response of the nictitating membrane even with small doses (⅙ of the dose

necessary to paralyze respiratory movements), while its administration during stimulation of the postganglionic fibers had no effect on the response even with doses double that necessary to paralyze respiration (fig. 5). Thus the effect of Erythrina is the same as that obtained in this preparation with curare by Luco and Mesa (4).
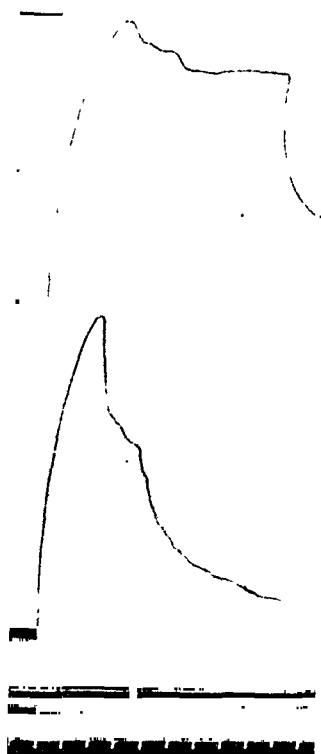
FIG. 5. Nictitating membrane. Simultaneous stimulation of the preganglionic fibers on one side (lower tracing) and the postganglionic fibers on the other side (upper tracing), in the same animal. Upper signal: the mark indicates the injection of 0.20 cc. of Erythrina. Middle signal: duration of the electric stimulation. Lower signal: 1 min. intervals.

2. *Stimulation by acetylcholine:* As described by Rosenblueth and Cannon (5), intraarterial injections of acetylcholine produce a double response of the nictitating membrane. The earlier part of the response (noted by an arrow in figure 6) corresponds to the excitation of the membrane by postganglionic nerve impulses set up by the acetylcholine in the ganglion, while the later part corresponds to the stimulation of the membrane itself by acetylcholine arriving via the blood stream. As may be seen in figure 6, the injection of Erythrina in doses of 0.10 cc. ($\frac{1}{3}$ of the dose necessary to paralyze the respiration) produces an intense depression of the ganglionic response without significantly affecting the response of the membrane

to the acetylcholine. The same was true even with larger doses in other experiments.

C. *Pupil.* 1. *Stimulation of the preganglionic parasympathetic fibers·* The stimulation of preganglionic parasympathetic fibers, like that of preganglionic sympathetic fibers (see Section B 1, and fig 5), produces a response whose amplitude falls rapidly to a lower level, which is maintained for some time (Luco and
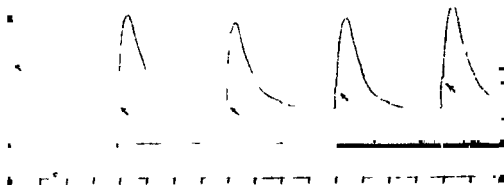
Fig 6 Effect of Erythrina on the superior cervical ganglion and the nictitating membrane stimulated by acetylcholine The arrows on the tracing point to the peaks of the early parts of the responses, corresponding to stimulation of the ganglion Upper signal each mark represents the injection of 40 micrograms of acetylcholine Middle signal injection of 0 10 cc of Erythrina Lower signal 1 min intervals
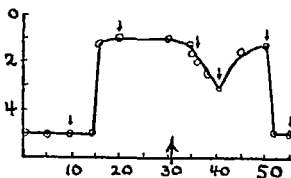
The arrows on the curve show, respectively, the times at which the above photographs were taken

Salvestrini (2)). During this stable period in the response, injections of Erythrina in doses of 0 10 to 0 90 cc. (⅓ to triple the dose necessary to paralyze respiration) produces a rapid depression, even with the smallest doses used.

2. *Stimulation of the postganglionic sympathetic and parasympathetic fibers·* The administration of Erythrina during stimulation of the postganglionic parasympathetic fibers causes a decrease of the pupillary constriction (figs. 7 and 8).

Its administration during stimulation of the postganglionic sympathetic nerves does not affect the pupillary dilatation (fig. 8).

These results agree with those observed by Luco and Mesa (4) on administering curare in the same preparation except with respect to the doses of the drugs. The depressing dose of curare was 3 to 5 times the dose of curare necessary to paralyze respiratory motions. With Erythrina, however, depression occurs with a dose of only $\frac{3}{4}$ of that which paralyzes respiration. As with curare, there was never a complete dilatation of the pupil when Erythrina was injected during postganglionic parasympathetic stimulation even when the doses were 3 to 4 times the dose necessary to paralyze respiration.
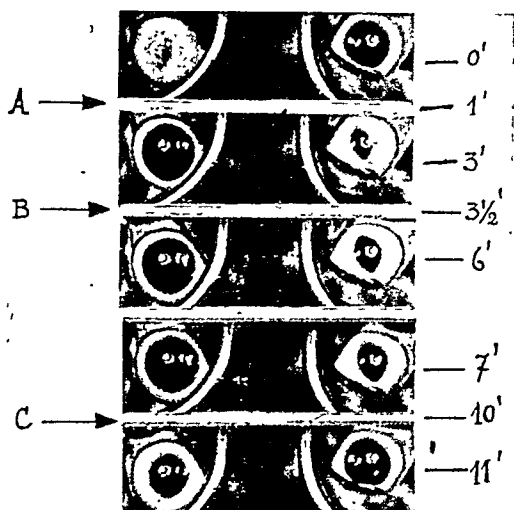
FIG. 8. Stimulation of the sympathetic and parasympathetic postganglionic fibers in the same animal. On the right, time in minutes. On the left, A and C indicate beginning and end of the stimulation; B, injection of 0 30 cc. of alcoholic extract of Erythrina.

D. *Submaxillary gland.* 1. *Stimulation of the chorda tympani (preganglionic parasympathetic fibers)*: The injection of Erythrina in various doses produced complete or partial curarization of the ganglion, according to the dose injected (figs. 9 and 11). The injection of prostigmine without Erythrina had no effect on the gland, but prostigmine injected during Erythrina depression caused a decurarization similar to that seen in the neuromuscular junction.

2. *Stimulation by acetylcholine:* Contrary to previous observations, small doses of Erythrina elicited a clear increase in the response of the gland (figs. 10 and 11). If, however, the first small dose was repeated at short intervals, the subsequent doses produced a typical paralysis, partial or complete, according to the total dose (fig. 10). The injection of a large dose ($\frac{1}{2}$ to $\frac{3}{4}$ of that necessary to paralyze respiration) could produce either an initial facilitation followed by a depression, or a pure depression even to complete curarization (fig. 10). Block with curare in this gland was demonstrated by Luco and Altamirano (6).

The potentiation demonstrated in these experiments may explain the intense salivation observed by Lehman, Chase, and Yonkman (7) in animals which had received Erythrina alkaloids.

The curarization observed either with stimulation of the chorda tympani or with acetylcholine was decreased or abolished by prostigmine (figs. 9 and 11).
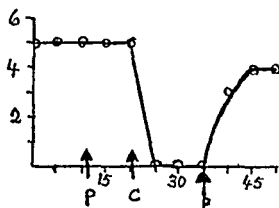
FIG 9 Secretion from the submaxillary gland on stimulation of the chorda tympani. Ordinates drops falling from a cannula placed in Wharton's duct. Abscissae time in minutes

Each arrow marked P indicates injection of 10 micrograms of prostigmine C, injection of 0 10 aqueous solution of Erythrina
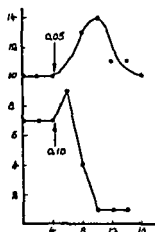
FIG 10
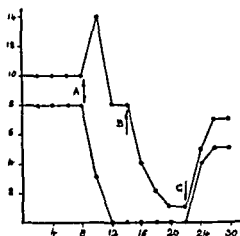
FIG 11

Fig 10 ... of Er ... 'choline Ordi- me in minutes ct of Erythrina

acetylcholine ijections affect both responses A and B, respectively, 0 05 and 0 20 cc of aqueous solution of Erythrina C, 10 micrograms of prostigmine

DISCUSSION: The simple method of extraction used with the Erythrina seeds makes it impossible to attribute the effects described to a single alkaloid.

In some experiments the alcoholic extract was used, and in others an aqueous solution of the residual after distillation of the alcoholic extract. This is of especial interest in the submaxillary gland, since Ettinger, Brown, and Megill (8)

report a lowering of the threshold to acetylcholine on the injection of alcohol. Their finding was confirmed in a series of experiments in which the same concentration of alcohol was injected as occurs in the alcoholic extract of Erythrina (6 to 8%). The response of the gland to injections of acetylcholine was augmented. In order to avoid this potentiating effect of alcohol, the experiments were repeated with an aqueous solution of Erythrina. The result was the same as described in Section D 2.

The effectors studied may be classified, after Dale, into one group innervated by cholinergic nerves and another, innervated by adrenergic nerves. The former group may be subdivided into two groups, nicotinic and muscarinic.

    I. Nicotinic effectors used:
        A. Skeletal muscle (Section A).
        B. Sympathetic ganglion (Section B).
        C. Parasympathetic ganglion (Sections C 1, and D 1).
    II. Muscarinic effectors used:
        A. Pupillary constrictor muscle (Section C 2).
        B. Salivary gland (Section D 2).
    III. Adrenergic effectors:
        A. Nictitating membrane (Section B 1).
        B. Pupillary dilator muscle (Section C 2).

Whether the nicotinic effectors were stimulated by acetylcholine or through their nerves, their responses were always depressed (curarization) by Erythrina.

When the pupillary constrictor was stimulated via its nerves, the effect of Erythrina was pure depression. In the salivary gland stimulated by injected acetylcholine, however, it produced first potentiation and then depression.

In the adrenergic effectors the doses of Erythrina used did not modify the responses to stimulation via the nerves.

It is apparent that the alkaloids of Erythrina affect the organs stimulated by acetylcholine and do not affect those stimulated by sympathin. In this respect these alkaloids are like curare, which was shown by Luco and Mesa (4) to act only on effectors innervated by cholinergic nerves, irrespective of whether they were nicotinic or muscarinic.

Further similarities to curare are found in the antagonism of prostigmine to Erythrina (fig. 2) and in the typical post-tetanic decurarization seen during the Erythrina depression (fig. 3). In addition, Erythrina, like curare (Rosenblueth and Luco (9)), raises the threshold of the effectors to acetylcholine.

All these considerations suggest that the mechanisms of the two drugs are the same, despite the fact that Harris and Harris (10) have shown that they react differently with cholinesterase.

Besides the depressive action, Erythrina has a facilitating effect on the salivary gland stimulated by injected acetylcholine, but not when stimulated via its nerves. This difference may depend on the fact that autonomic nerve endings frequently penetrate to the interior of the effector cells, while injected acetylcholine arrives at their external surfaces. The facilitating action may depend on the function of the cellular membrane.

Could this facilitating effect depend on an improvement of the local circulation? This explanation might be favored because Erythrina produces a fall of arterial pressure (Cicardo and Hug (1)) It can, however, be excluded on the basis of the following considerations 1 The doses used are small compared to those which cause hypotension, 2 repeated injections of acetylcholine produce a continual change in the circulation of the gland without altering the response to the injected acetylcholine, 3 the injection of prostigmine (see figure 9), also a hypotensive drug, does not change the response to acetylcholine, and 4 the frequent injections into the carotid artery, which surely provoke changes of pressure in the effector, do not modify the responses

The double effect of Erythrina recalls the double effect of prostigmine or nicotine on nicotinic effectors, but the alkaloids of Erythrina have this double effect on muscarinic effectors

SUMMARY

The effects of alcoholic and aqueous extracts of Erythrina crista galli (ceibo) were studied in cats anesthetized with dial

These extracts produce a depression of the responses of effectors innervated by cholinergic nerves when these effectors are stimulated by injected acetylcholine or through their nerves In the doses used, they do not affect the organs innervated by adrenergic nerves

This curarizing effect is antagonized by prostigmine (fig 2) and, at the neuromuscular junction, also by the application of a tetanus (post tetanic decurarization, fig 3)

Besides the curarizing effect, it is shown that in the submaxillary gland stimulated by acetylcholine small initial doses of Erythrina provoke a clear increase of the response and large doses cause a decrease (figs 10 and 11) In some cases a moderate dose could produce both effects, first potentiating and then depressing

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THE DISTRIBUTION OF RADIANT ENERGY IN THE FLUORESCENT SPECTRA OF ATABRINE AND SOME OTHER DERIVATIVES OF ACRIDINE[1]

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These quantitative studies of fluorescent spectra were undertaken because the information was considered desirable in connection with the use of fluorometric methods for the determination of atabrine and because it was hoped that the comparison of fluorescent spectra might be helpful in the identification of degradation products of atabrine in biological materials.

METHODS. The intensities in the fluorescent spectra were measured by a photographic method. The general principles on which this method is based are explained in detail in articles by Harrison (1, 2) and by Jones (3).

Fluorescence was excited by the light of a mercury arc passed through Corning filters 5840 and 738. The 365 mμ line is the only strong line of the mercury spectrum passed by these filters.

All of the solutions were at a temperature of about 25°. They were contained in rectangular glass cuvettes. The spectrum of the fluorescent radiation emerging at right angles to the exciting light was photographed with a prism spectrograph.

For the preliminary determination of the shape and extent of the spectra, they were usually photographed on Eastman Super-XX cut film, which was found suitable for this purpose because of its high speed, medium contrast, and wide latitude. The regions of peak intensity were redetermined on Eastman Spectrographic Plates type III-B or 103-B, which have emulsions of much higher contrast.

For the calibration and standardization of the photographic emulsion, a tungsten filament lamp operating at a color temperature of 2800°K was used. The energy distribution of this source was assumed to be that given by Planck's Law for a black body at that temperature. The intensity of light from the standard source was varied by means of a rotating sectored disk of adjustable aperture delivering 50 flashes per second. In view of the work of Webb (4), this frequency was considered high enough to make any reciprocity-intermittency errors unlikely. On each film or plate were photographed one or more fluorescent spectra together with a series of spectra of the standard source at different intensities.

The densities of the silver deposits were measured with a photoelectric microphotometer and the intensity of the unknown spectrum at any wavelength determined by a process of interpolation.

The absorption spectra were measured with a Cenco-Sheard "Spectrophotelometer".

The fluorescent spectra and the adjacent portions of the absorption spectra are shown in the charts. The absorption spectra are shown by the dotted lines and the fluorescent spectra by the solid lines. In each fluorescent spectrum the maximum intensity found in that spectrum is arbitrarily represented as 100 and
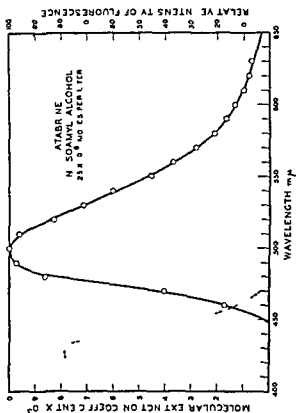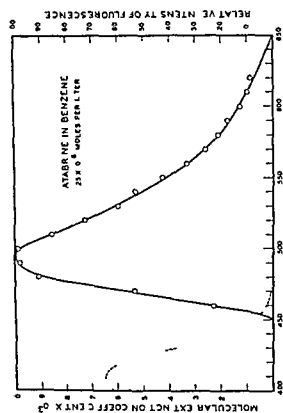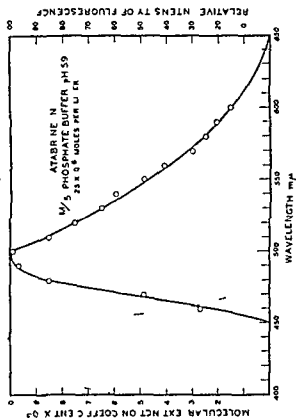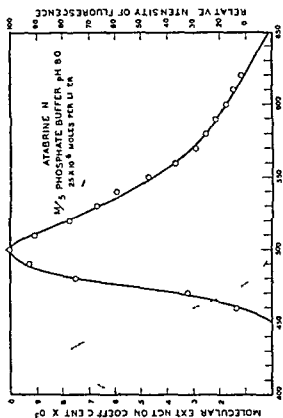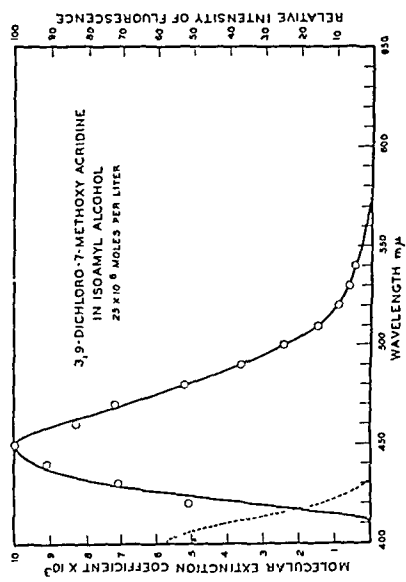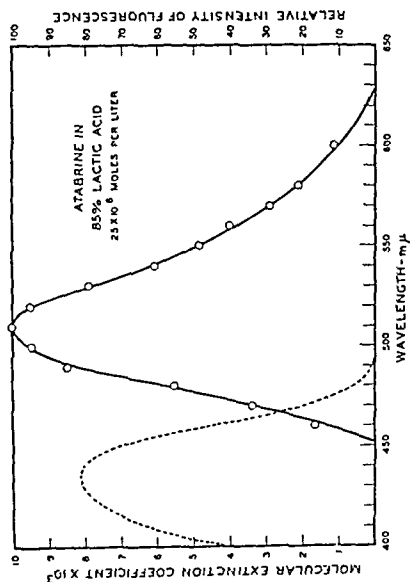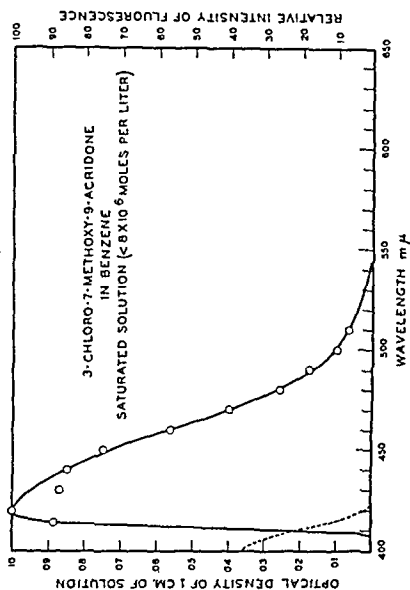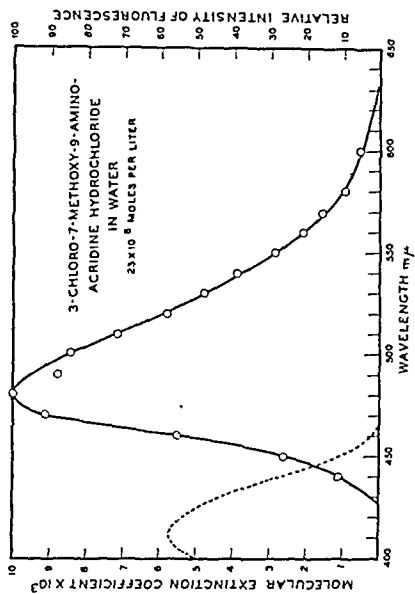
the radiant intensities at other wavelengths are expressed relative to that. The peak intensities of the eight fluorescent spectra studied stand in approximately the following ratios. (These ratios are not precise. The exposures were necessarily made at different times and for different lengths of time. The calculation of the ratios therefore involves the assumptions of the constancy of the exciting light and the validity of the reciprocity law for the photographic emulsion.)

Atabrine in phosphate buffer pH 5.9	1.0
Atabine in phosphate buffer pH 8.0	6.4
Atabrine in benzene	5.2
Atabrine in isoamyl alcohol	17.0
Atabrine in lactic acid	8.5
3-Chloro-7-methoxy-9-amino acridine	65
3,9-Dichloro-7-methoxy acridine	65
3-Chloro-7-methoxy-9-acridone	47

The form of all of these fluorescent spectra and their relation to the absorption spectra conform to the description given by Kauffmann (5) for other fluorescent dyes. That is, the fluorescent spectrum covers a wide band of longer wavelength than the absorption band but adjacent to it with some overlapping.

Although the solvent affects the intensity of atabrine fluorescence to a large extent, it has little influence on the relative distribution of energy in the fluorescent spectrum. The curves for atabrine in the first four solvents listed above scarcely differ by more than the experimental error. In lactic acid the peak intensity has shifted to a slightly longer wavelength. The curves for the other three acridine derivatives are of the same general shape, but the peak intensities are at shorter wavelengths.

In each of the eight cases studied here, any wavelength of light within the absorption band excites fluorescence. The maximum exciting wavelength corresponds closely with the point at which the absorption band fades out. Thus 450 mμ will excite atabrine in benzene but not the acridone in benzene.

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FORMATION OF METHEMOGLOBIN

III. THE INFLUENCE OF TOTAL HEMOGLOBIN ON THE FORMATION OF METHEMOGLOBIN FROM ACETANILIDE

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In a previous paper of this series (1), it was shown that the administration of acetanilide in doses of 1, 2 and 3 grams daily for 6 weeks did not lead to an accumulation of methemoglobin. The methemoglobin formed by the acetanilide given one day disappeared before the next, and the amount formed from the drug given on the succeeding day was no greater than on the preceding. With the two larger doses of acetanilide used, there was a moderate but progressive decrease in total hemoglobin; the average for 29 subjects fell from 13.0 to 11.3 g. per 100 cc. in the course of a month. Since the amount of methemoglobin formed at the daily determination remained virtually constant, the per cent increased to the extent that the total hemoglobin decreased. The findings of these experiments suggest, but by no means establish, the possibility that the amount of methemoglobin formed is independent of the total hemoglobin. Such a possibility would imply a definite limitation to the administration of acetanilide in anemia. Thus, if the amount of methemoglobin formed were independent of the total hemoglobin, the residual active hemoglobin would be decreased correspondingly with the anemia and any anoxemic effect would be proportionately exaggerated. This possibility has sufficient clinical importance to justify an investigation of the relation of methemoglobin formation to total hemoglobin.

Cats were used as the experimental animal because of their sensitivity to methemoglobin formation. This sensitivity was particularly desirable since, with the dose of acetanilide necessary to produce large amounts of methemoglobin in less sensitive animals, such as dog and man, or, still more so, the rat, delay and variation in absorption introduce serious complications.

Fifty-four experiments were carried out on 14 cats. The total hemoglobin of the animals ranged from 5.0 to 11.6 g. per 100 cc.; 4 of the animals were anemic initially and the remainder were rendered so, as desired, by daily withdrawal of blood. Acetanilide, suspended in gum acacia, was given by stomach to the fasted animals in doses of 10, 20, 35 and 50 mg./kg. The methemoglobin was determined (2) at 30 minute intervals until the maximum amount was reached and passed. Figure 1 shows the maximum concentrations at various doses of acetanilide in relation to the total hemoglobin. With a dose of 10 mg./kg. there was, as in the human experiments mentioned in the first paper of this series, little relation between the small amounts of methemoglobin formed and the total hemoglobin. The residual hemoglobin was therefore nearly directly proportional to the total. In contrast, with a dose of 20 mg./kg., the amount of methemoglobin varied with the total hemoglobin; and with doses of 35 mg./kg.

and larger, the amount of methemoglobin formed was expressed by the relation $Met = total\ Hb - k$. The value k, which corresponds to the residual hemoglobin, averaged 2 8 g with extremes of 2 1 and 3 2. Thus with doses of acetanilide large
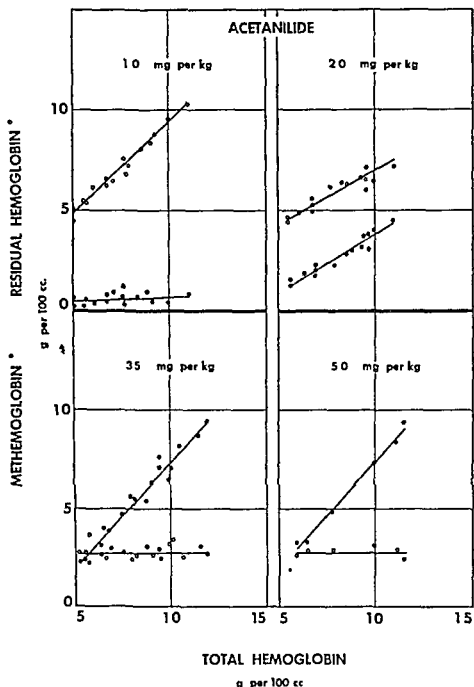
FIG 1 RELATION OF METHEMOGLOBIN AND RESIDUAL HEMOGLOBIN TO TOTAL HEMOGLOBIN AFTER VARIOUS DOSES OF ACETANILIDE

enough to produce considerable amounts of methemoglobin the formation varies with the total hemoglobin but ceases at a residual hemoglobin sufficient to support the life of the animal

The limitation to methemoglobin formation from larger doses of acetanilide is

further shown by data presented in figure 2. A cat was given acetanilide in doses of 10, 20, 35 and 50 mg./kg. at intervals of 1 week over a period of several weeks and the maximum methemoglobin formation on each occasion was recorded. The animal was then bled until the total hemoglobin was reduced to 8 to 9 g. and was maintained at that level; the 4 doses of acetanilide were then given at intervals and the maximum amounts of methemoglobin formed again determined. The entire procedure was repeated at a total hemoglobin of 5 to 6 g. With a dose of 10 mg./kg. there was little difference in the maximum amount of methemoglobin formed; with larger doses, the differences were wide. With 50 mg./kg., 8.2 g. of methemoglobin were formed at a total hemoglobin of 11 to
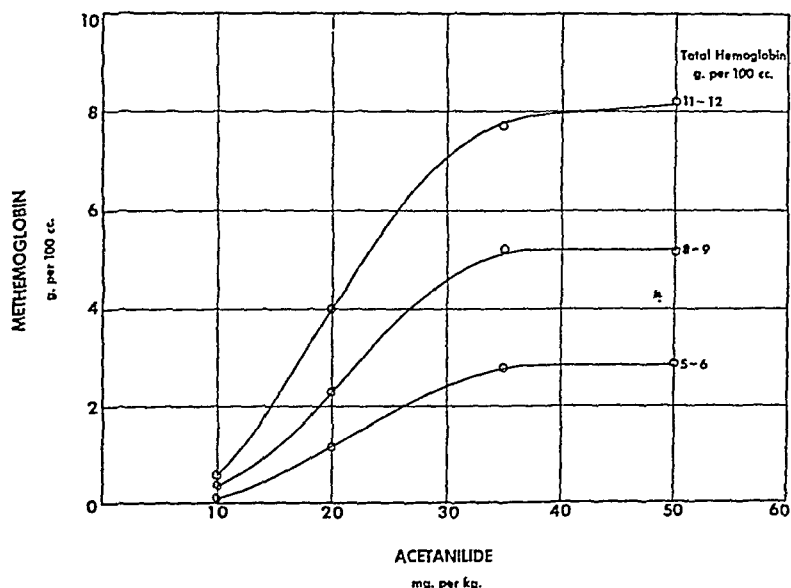
FIG. 2. MAXIMUM FORMATION OF METHEMOGLOBIN FROM ACETANILIDE AT DIFFERENT LEVELS OF TOTAL HEMOGLOBIN

12 g.; 5.2 g. at a total hemoglobin of 8 to 9 g.; and 2.7 g. at a total hemoglobin of 5 to 6 g. The residual hemoglobins were respectively 3.3, 3.2 and 2.8 g.

In view of the fact that with a dose of acetanilide sufficient to cause considerable formation of methemoglobin this formation varies with the total hemoglobin and that a minimal residual hemoglobin remains, irrespective of the amount of total hemoglobin, it would appear that anoxemia from methemoglobin formation from acetanilide is no more severe in the presence of anemia than in that of a normal amount of hemoglobin. It would appear further that because of the limitation to the formation of methemoglobin, methemoglobin itself is not a primary factor in any toxicity that this drug may have, and that in any one

species of animal the extent of methemoglobin formation is not a measure of the toxic action of the drug. The animals used in this study survived doses of acetanilide which formed the maximum amount of hemoglobin, but could be killed by massive doses which produced no greater amounts. Cats with high total hemoglobins gave a much greater amount of methemoglobin than did anemic cats, but required as large doses to kill as did the anemic cats.

Acetanilide does not produce methemoglobin *in vitro*. In the living animal, the methemoglobin from acetanilide results from metabolites of the acetanilide which are subsequently destroyed or eliminated. The fact that *in vivo* the formation of methemoglobin is, for larger doses, and up to the limit of formation, proportional to the total hemoglobin implies either that the formation or destruction of the metabolite responsible for methemoglobin is influenced by the total hemoglobin, or, that the formation of methemoglobin by the metabolite is influenced by the total hemoglobin.

CONCLUSIONS

(1) With small doses of acetanilide, the small amounts of methemoglobin formed are independent of the total hemoglobin.

(2) With large doses, the amounts of methemoglobin formed are proportional to the total hemoglobin, but, even with very large doses, the formation ceases at a residual hemoglobin sufficient to support life.

(3) Methemoglobin formation is not a primary factor in the toxicity of acetanilide.

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FORMATION OF METHEMOGLOBIN

IV. LIMITED IMPORTANCE OF METHEMOGLOBINEMIA IN THE TOXICITY OF CERTAIN ANILINE DERIVATIVES

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The formation of methemoglobin following administration of aniline and certain aniline derivatives, such as acetanilide, is so obvious an effect that it has often been regarded as an important and even the main feature in the toxicity of these drugs. This belief is not supported by any evidence; on the contrary, the comparative toxicities of acetanilide for single and repeated doses in animals of different species does not correspond to the order of sensitivity of these animals to methemoglobin formation (1, 2, 3). The results of the investigation reported here would indicate that the toxic effects are exercised by the aniline derivative itself or by its metabolites and that the formation of methemoglobin is not an important feature in the toxicity.

The drugs used here were those proposed as intermediary products of the metabolism of aniline and as the active agent in producing methemoglobin: β-phenylhydroxylamine and p-aminophenol. The toxic effects and maximum formation of methemoglobin were determined in rats for a range of doses extending to the lethal.

Total hemoglobin and methemoglobin were determined by the method of Evelyn and Malloy (4) on blood samples taken before and at intervals of 30 to 60 minutes after administration of the drugs. A curve for methemoglobin formation was plotted for each animal following administration of the drug and the maximum formation taken from the curve (1). Methemoglobin is expressed as per cent of total hemoglobin; the total hemoglobin in all animals used was the same within ± 0.5 g. and averaged 15 g. per 100 cc. of blood. The drugs were administered in water solutions by intraperitoneal injection. The β-phenylhydroxylamine was prepared by the method of Bamberger (5) and kept from deteriorating by maintaining it under nitrogen and at a low temperature; and the p-aminophenol was given as the hydrochloride.

The extent of methemoglobin in respect to the doses of the drugs administered is shown in figure 1 in which the abscissae indicate the dose of drugs given and the ordinate the maximum formation of methemoglobin in the animals given these doses.

As shown previously for acetanilide (6), the amount of methemoglobin formed increased with increasing doses of both drugs but reached a maximum after which further increase of dose caused no greater formation of methemoglobin. With p-aminophenol, the maximum formation of methemoglobin was 30 to 35 per cent; with β-phenylhydroxylamine, 63 to 66 per cent. This maximum formation for any one species of animal thus appears to be a characteristic of the drug. The

maximum, as defined here, may possibly be used to give some indication of the nature of the intermediary products from the metabolism of aniline itself. Thus in one experiment 300 mg. per kg. of aniline hydrochloride was given to a rat and the maximum formation of methemoglobin was 47 per cent. This maximum is above that which can be obtained with p-aminophenol and therefore suggests that if p-aminophenol is a product of the metabolism of aniline it is not the only methemoglobin-forming metabolite.

The lethal dose for β-phenylhydroxylamine (Fig. 1) was 35 mg. per kg.; and for p-aminophenol, 640 mg. per kg. The maximum formation of methemoglobin
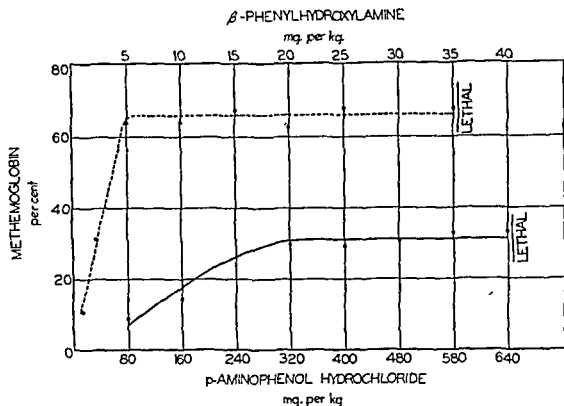
FIG. 1. EXTENT OF METHEMOGLOBIN FORMATION FROM VARIOUS DOSES OF
P-AMINOPHENOL AND β-PHENYLHYDROXYLAMINE
Lethal doses are indicated

occurred with one-seventh the lethal dose of β-phenylhydroxylamine and with one-half of that of p-aminophenol. At the smallest dose producing maximum formation of methemoglobin, 5 mg. per kg. β-phenylhydroxylamine and 320 mg. per kg. p-aminophenol, the only symptom observed in the animals was some depression which quickly passed. As the doses were increased—but with no further increase in formation of methemoglobin—toxic effects appeared and became progressively more marked as the lethal dose was approached. In death from these drugs, cardiac and respiratory failure occurred nearly simultaneously; in asphyxiation, as from the formation of carbon monoxide hemoglobin, cardiac failure follows respiratory failure by several minutes.

The facts (a) that a maximum formation of methemoglobin occurs from

β-phenylhydroxylamine and p-aminophenol at doses of these drugs which produce no appreciable toxic effects or evident symptoms of anoxemia and (b) that increase of the dose is attended with symptoms of drug intoxication and death but with no further formation of methemoglobin, give support to the belief that methemoglobin formation plays little part in the toxicity of these and kindred drugs. The formation of methemoglobin may be looked upon as a secondary or side reaction, limited by a definite maximum which varies with the drug and the species of animal. Aside, therefore, from the imposition which large amounts of methemoglobin may place upon the respiratory functions of the blood and which were not grossly evident for the largest amounts reached in the experiments reported here, methemoglobin formation appears to be unrelated to the acute toxicity of these drugs.

CONCLUSIONS

The formation of methemoglobin plays no important part in the acute toxicity of β-phenylhydroxylamine and p-aminophenol. Indications are given that this fact applies also to aniline and acetanilide.   .

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THE DETOXICATION OF NEOARSPHENAMINE BY MEANS OF VARIOUS ORGANIC ACIDS

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It was first pointed out by Ehrlich (1) that one of the most important means of detoxifying chemotherapeutic agents, particularly the arsphenamines, is by chemical reduction A number of organic acids might be expected to exert this reducing action Ascorbic acid occupies a unique position in this connection because it is a powerful reducing agent and is normally found in animal tissues Dainow (2) and Landfisch (3) almost simultaneously recommended that ascorbic acid be added to solutions of neoarsphenamine in order to prevent changes in toxicity resulting from oxidation by the air That excessive exposure to air results in a considerable increase in toxicity is well known (4) and is the basis for the precautions taken in preparing such solutions (5)

Recently Bundesen, Aron, Greenebaum, Farmer, and Abt (6) reported that the usual darkening of solutions of Mapharsen (the hemialcoholate of 3 amino 4 hydroxyphenylarsine oxide hydrochloride) and neoarsphenamine on exposure to air does not occur if 10% of ascorbic acid is present They also found that the usual skin reactions (patch test) to 30% solutions of neoarsphenamine were practically eliminated if ascorbic acid in a concentration of from 10 to 20% were added to the solution being tested These authors have given a comprehensive review of the literature, and no further general comment seems required beyond reference to more recent work

Rosenthal (7) has shown that the oral toxicity of Mapharsen is markedly decreased when it is accompanied by from 2 to 5 moles of glutathione Cysteine, on the other hand exerted no demonstrable protective effect

Sandground and Hamilton (8) have shown that p aminobenzoic acid exerts a marked detoxifying effect on a number of pentavalent arsenical compounds This applied regardless of the route of administration, the arsenical was never given by the same route as the p aminobenzoic acid A quantity of 15 mgm / kgm of the acid was sufficient to protect 50% of rats against LD$_{90}$ of the pentavalent arsenicals studied (9) Since p aminobenzoic acid obviously cannot act as physiological reducing agent, it must act by some other mechanism Sandground (10) has suggested that the mechanism may be explained along the lines of the enzyme blockade theory This hypothesis was based upon the fact that p aminobenzoic acid conferred protection upon the animals if given at any time up to three hours before the arsenical, but its effectiveness was greatly reduced if given more than thirty minutes after the arsenical The same paper also referred to a protective action of p aminobenzoic acid against neoarsphenamine

In most of the published work, ascorbic acid has been administered in the same solution as neoarsphenamine when a protective effect was desired. However, Martin and Johnson (11) have reported that ascorbic acid is most effective in protecting mice against the toxic effects of neoarsphenamine when it is injected about two hours before the arsenical, in order to permit complete diffusion in the tissues.

The purpose of the present work has been to study further the detoxifying action of several organic acids, particularly ascorbic acid, on neoarsphenamine with a view toward elucidating the mechanism and physiological effects.

EXPERIMENTAL METHODS. All animals used as subjects in these experiments were albino rats from our own stock, weighing from 80 to 120 gms. and from 35 to 50 days of age. They were fasted for 16 hours previous to the injections, which were made into the saphenous vein in the usual manner, using 8% solutions of neoarsphenamine prepared 15 minutes before injecting the first animal (5). The dosages ranged from 400 to 450 mgm./kgm. depending somewhat on the purpose of the experiment and the toxicity of the preparation. For the toxicity studies, the animals were observed up to 6 days after the injection.

For tissue respiration studies, the organs were removed immediately after the sacrifice of the animal and transferred to saline. Thin slices were then cut, blotted gently on filter paper, weighed and transferred to the Warburg apparatus. The respiration of the tissue in glucose-phosphate buffer was measured in the usual way.

For histological examination the liver, kidney, and spleen were removed from the animals immediately after sacrifice. The organs were fixed in Zenker's fluid, and, after the usual process of preparation, were stained with haematoxylin-eosin.

Blood for the chemical studies was collected (and oxalated) following decapitation of the animals. Sufficient whole blood (2 cc.) was immediately removed for the arsenic analysis, after which the tube was stoppered and the plasma was separated by centrifugation. At the time of sacrifice, the livers and kidneys of the animals were removed for arsenic analysis. Plasma ascorbic acid was determined by the method of Mindlin and Butler (12). Arsenic determinations were made by the method of Maechling and Flinn (13).

A different group of animals was used for each of the different studies mentioned above.

Effect of various organic acids on the toxicity of neoarsphenamine. The animals received a 8% solution of neoarsphenamine in sufficient quantity to represent 450 mgm./kgm. This dosage usually kills 75% or more of the animals of our colony within 6 days, with characteristic renal changes. The various organic acids were also administered at a level of 450 mgm./kgm., sufficient sodium carbonate being added to convert the acid to its sodium salt (the organic acid and the arsenical were dissolved in the same solution). Because the study was to include a considerable number of acids, these preliminary experiments were conducted with only 4 animals to each group. The acids studied included: ascorbic, isoascorbic, d-glucoascorbic, lactic, pyruvic, succinic, malic, mandelic, aspartic, gluconic, 2-ketogulonic,[1] and l-cysteine. Positive protection was obtained only with the three ascorbic acids and lactic acid. Evidence of protection was based primarily on survival of the animals up to 6 days after the injections, but the observations were further fortified by autopsy of the surviving animals. The protected animals were practically free from gross pathological lesions.

[1] This compound readily lactonizes in acid solution to form ascorbic acid.

These preliminary experiments indicated that only the most labile physio logical reductants are effective detoxicants (cysteine is an exception) It then became of interest to determine the minimum level at which they exert a definite protective effect For this purpose neoarsphenamine was administered at levels so chosen that about three fourths of the control animals would fail to survive for 6 days This level depended on the toxicity of the preparation used The organic acid was at first administered with the neoarsphenamine in about an

TABLE 1

Mortality of albino rats receiving neoarsphenamine with various amounts of ascorbic or lactic acid (as the sodium salt)

ORGANIC ACID	mM PER KG		mM ACID PER mM NEOARSPHENAMINE	MORTALITY
	Acid	Neoarsphenamine		
None		0 91	0	5/6
Lactic	5 00	0 91	5 10	0/6
Lactic	2 73	0 91	3 00	1/6
Lactic	1 36	0 91	1 50	4/6
Ascorbic	2 73	0 91	3 00	0/6
Ascorbic	1 36	0 91	1 50	0/6
Ascorbic	0 68	0 91	0 75	0/6
Ascorbic	0 44	0 91	0 50	0/6
Ascorbic	0 22	0 91	0 25	2/6
None		0 86*	0	2/6
None		0 97	0	4/6
None		1 03	0	5/6
Ascorbic	1 03	1 03	1 00	0/11
Ascorbic	1 20	1 20	1 00	4/12
Ascorbic	1 37	1 37	1 00	7/15
Ascorbic	1 54	1 54	1 00	5/6
Ascorbic	1 54	1 03	1 50	1/11
Ascorbic	1 80	1 20	1 50	0/11
Ascorbic	2 06	1 37	1 50	7/14
Ascorbic	2 31	1 54	1 50	5/6
Ascorbic	3 09	1 03	3 00	0/8
Ascorbic	3 60	1 20	3 00	0/11
Ascorbic	4 10	1 37	3 00	3/14
Ascorbic	4 62	1 54	3 00	2/6

* A different lot of neoarsphenamine was used for the experiments below the line

equal amount by weight, then the quantity of the acid was reduced stepwise until a level was found at which there was no longer complete protection of the animals (i e , 100% survival) The results, given in table 1, indicate that more than 3 moles of lactic acid are required to give complete protection against 1 mole of neoarsphenamine, while only about ½ mole of ascorbic acid is required for the same purpose The results below the line in the table (a different lot of the arsenical was used to obtain them) show that if the amount of ascorbic acid is increased beyond this ratio, some further decrease in toxicity is observed

Thus, the mortality of animals receiving 1.37 mM. of neoarsphenamine and 4.10 mM. of ascorbic acid per kgm. is not as great as in animals which received the same dose of neoarsphenamine and either 2.06 or 1.37 mM. of ascorbic acid per kgm.

In table 2 a comparison has been made of the relative effectiveness of ascorbic, isoascorbic, and d-glucoascorbic acids as detoxicants for neoarsphenamine. The procedures require no explanation since they were the same as described above. The arsenical was given at various levels, and the organic acid was given in the same amount by weight. The results indicate that the three acids may be used interchangeably and that antiscorbutic activity has, therefore, no bearing on detoxifying power.

The experiments thus far described throw little light on the manner in which the ascorbic acids act to detoxify neoarsphenamine beyond the fact that their

TABLE 2

Mortality of albino rats receiving various doses of neoarsphenamine and the same amount by weight of ascorbic (or isoascorbic or d glucoascorbic) acid (as the sodium salt)

DOSE	NEOARSPHENAMINE ONLY	NEOARSPHENAMINE WITH		
		Ascorbic acid	Isoascorbic acid	d Glucoascorbic acid
mgm /kgm				
360	1/10	0/8	0/8	
400	1/14	0/15	0/15	
440	5/13	0/20	0/20	0/6*
480	9/13	1/15	1/15	
560	10/10	2/13	0/13	2/6*
640		3/13	3/13	
720		5/10	6/10	

* On this test five of six control animals receiving 450 mgm /kgm of neoarsphenamine failed to survive

reducing action is the most important consideration. The question remains whether they would be as effective when administered by different routes, since in the above experiments both the neoarsphenamine and the organic acid were given intravenously in the same solution. Accordingly, the following experiment was carried out, using 6 animals to each group:

Group A. 450 mgm /kgm. neoarsphenamine injected intravenously. One animal survived six days

Group B. 450 mgm./kgm. neoarsphenamine and 100 mgm./kgm ascorbic acid (as the sodium salt) injected intravenously in the same solution. All animals survived.

Group C. 450 mgm /kgm neoarsphenamine injected intravenously and 200 mgm./kgm. ascorbic acid (as the sodium salt) intramuscularly immediately afterward. Three animals survived.

Group D. 200 mgm./kgm. ascorbic acid (as the sodium salt) injected intramuscularly followed two hours later by 450 mgm./kgm. neoarsphenamine intravenously. No animals survived.

The results indicate that ascorbic acid is maximally effective if given intravenously in the same solution as the neoarsphenamine. Its effectiveness is seriously reduced if it is administered simultaneously at another site (e g , intramuscularly) and is completely lost if administered at another site two hours before the neoarsphenamine. Evidently for effective detoxication the neoarsphenamine must be exposed to a high concentration of ascorbic acid. The total amount of ascorbic acid available to the animal is not a determining factor as is clearly shown by the difference between groups B and C.

While these experiments were in progress Sandground (14) reported a protective effect of p aminobenzoic acid against pentavalent phenyl arsonates and stibonates. This suggested that it would be worth while to supplement his studies by comparing the effectiveness of p aminobenzoic and ascorbic acids as detoxicants for neoarsphenamine. Three sets of 16 animals each were treated as follows

Group A   450 mgm /kgm of neoarsphenamine injected intravenously Three animals survived to the 6th day

Group B   450 mgm /kgm of neoarsphenamine injected intravenously, followed immediately by 132 mgm of p aminobenzoic acid (as the sodium salt) intramuscularly (The quantity of the acid chosen represents a molecular ratio of 1 1 with respect to neoarsphenamine) Seven animals survived to the 6th day [2]

Group C   450 mgm /kgm of neoarsphenamine and 132 mgm /kgm of p aminobenzoic acid (as the sodium salt) injected intravenously in the same solution (prepared 15 minutes before the first injection) Fifteen animals survived to the 6th day

There is evidently little to choose between ascorbic acid and p aminobenzoic acid. They are about equally effective when administered at a level of 1 mole for each mole of neoarsphenamine, and both are most effective when administered in the same solution as the neoarsphenamine

Effect of ascorbic (and isoascorbic) acid on the excretion of arsenic Groups of at least 10 rats were used for each of the chemical studies. They received 400 mgm /kgm of neoarsphenamine, or of ascorbic (or isoascorbic) acid, or of both the arsenical compounds and one of the acids. All injections were intravenous. Two animals were killed at each of the selected time intervals and the analyses were carried out by methods referred to above. In case a good agreement was not obtained in the figures from the two animals killed at the same time,

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[2] It might be argued that at the p aminobenzoic acid would have been more effective if the tissues were previously saturated with it. However we have found that the daily oral administration of 50 to 250 mgm /kgm of the acid for 3 days before the neoarsphenamine injection confers no protection on the animals. For example in a group of animals receiving the 250 mgm /kgm dose and from 360 to 420 mgm /kgm of neoarsphenamine the mortality was 8/10 while in a similar group of control animals the mortality was 5/10. Furthermore the inclusion of 0 25 or 0 50% of the acid in the diet beginning 3 days before the injection of neoarsphenamine (450 mgm /kgm) resulted in a mortality just as high as in control animals on the regular diet. These animals were not fasted for 16 hours before the injections as is usually done. Peters (15) was unable to protect mice against Mapharsen by the prior administration of p aminobenzoic acid

the experiments were repeated until a sufficient number of figures was available to indicate their statistical significance. The results are given in table 3.

The purpose of this work was to determine whether the simultaneous injection of ascorbic or isoascorbic acid causes any significant change in the blood levels

TABLE 3

Effects of ascorbic and isoascorbic acids on the blood levels and organ contents of arsenic following neoarsphenamine

Each substance administered at a level of 400 mgm./kgm.

MEDICATION	TIME AFTER MEDICATION	BLOOD LEVELS (MGM. %)				ORGAN CONTENTS AS MGM. As₂O₃ PER 100 GRAMS FRESH TISSUE			
		Ascorbic or isoascorbic acid		AS₂O₃		Liver		Kidney	
		Average	Range	Average	Range	Average	Range	Average	Range
	hours								
None		2.0	1.5– 3.1 (7)*						
Ascorbic acid	$\frac{1}{30}$	430	338 –572 (4)						
	$\frac{1}{2}$	122	100 –144 (2)						
	1	36	35.2– 36.8 (2)						
	6	12	3.4– 14.4 (4)						
	24	1.8	1.6– 1.9 (2)						
Isoascorbic acid	$\frac{1}{30}$	439	422 –456 (2)						
	$\frac{1}{2}$	158	133 –183 (2)						
	1	60	56 – 64 (2)						
	6	6	5 – 7 (2)						
	24	1.6	1.3– 1.8 (2)						
Neoarsphenamine	$\frac{1}{30}$			60	58 –67 (4)	18	16–20 (4)	69	50–105 (4)
	$\frac{1}{2}$			30	22 –38 (4)	22	13–28 (4)	71	50–100 (4)
	1			22	20 –23 (4)	22	14–27 (4)	54	35– 85 (4)
	6			10	7 –12 (2)	30	30 (2)	58	57– 60 (2)
	24			5	5 (1)†	12	9–14 (3)	56	56 (1)†
Neoarsphenamine and ascorbic acid	$\frac{1}{30}$	415	372 –434 (4)	50	48 –53 (2)	19	16–21 (4)	66	42–105 (4)
	$\frac{1}{2}$	150	142 –155 (3)	24	19 –29 (2)	27	22–30 (4)	63	50– 70 (4)
	1	65	37 – 92 (4)	21	17 –23 (2)	22	18–26 (4)	44	27– 48 (4)
	6	55	16 – 96 (4)	9	8 –10 (2)	32	30–37 (4)	47	31– 60 (4)
	24	15	2 – 35 (4)	3	3 – 4 (3)	17	9–20 (4)	33	16– 56 (4)
Neoarsphenamine and isoascorbic acid	$\frac{1}{30}$	472	456 –489 (2)	52	46 –60 (2)	24	21–27 (2)	53	50– 55 (2)
	$\frac{1}{2}$	203	150 –266 (4)	25	22 –27 (4)	31	30–31 (2)	40	30– 50 (2)
	1	104	87 –180 (3)	18	16 –20 (3)	31	28–33 (2)	110	90–130 (2)
	6	85	14 –160 (4)	10	7 –12 (4)	31	22–39 (2)	29	25– 33 (2)
	24	10	1.7– 43 (4)	6	4.5– 7 (5)	24	22–26 (2)	37	22– 52 (2)

* Figures in parentheses represent number of animals in the groups.

† Duplicate failed to survive 24 hours.

of arsenic after neoarsphenamine. Although the number of animals used was not very great, it appears that there is no important difference in the blood levels of arsenic at various time intervals in the neoarsphenamine animals as compared to the levels in those animals which received ascorbic or isoascorbic acid as well. The analysis of liver and kidney for arsenic at the various time intervals also failed to show any noteworthy difference from the controls.

However, the excretion of the ascorbic acids was somewhat delayed by the simultaneous administration of neoarsphenamine

TABLE 4

The effect of various quantities of ascorbic (and p aminobenzoic) acid on the toxicity of neoarsphenamine as evidenced by the respiration of kidney slices

MEDICATION (MGM /KGM)		DAYS AFTER MEDICATION	KIDNEY RESPIRATION (CMM O₂ PER 100 MGM [MOIST] PER HOUR)
Neoarsphenamine	Ascorbic acid*		
0	0		335§
400	0	1	212
400	0	2	138
400	0	3	131
400	0	4	152
400	0	5	209
0	200	1	368
0	200	3	330
400	100	1	300
400	100	2	266
400	100	3	310
600	150	1	314
600	150	2	335
600	150	3	300
400	50	1	274
400	50	2	244
400	50	3	186
400	50	4	259
425	200†	2	185
425	200†	3	170
425	125‡	2	275
425	125‡	3	340
450	132†‡	4	275
450	132†‡	5	302

* All injections intravenous except where otherwise noted

† Intramuscular injections, given immediately after the neoarsphenamine

‡ p aminobenzoic acid

§ Each figure represents the average of three or four tissue slices from the same animal The standard error of the determinations was 15–20%

Respiration of kidney slices following injections of neoarsphenamine and ascorbic acid Since the kidney is the organ most affected by neoarsphenamine (16), respiration of kidney slices from treated animals was studied The animals were injected in the usual way and studied at daily intervals as indicated in table

4. The results support the general picture presented by the toxicity studies. Kidney respiration is definitely depressed by neoarsphenamine, particularly on the second to fourth days after treatment although biological variability cannot be ruled out as accounting for some of the differences noted at the various time intervals. Animals which also received ascorbic acid intravenously show essentially normal kidney respiration values except in the case of those which received only 50 mgm./kgm. Here evidently the toxic effects of the neoarsphenamine were not entirely overcome. P-aminobenzoic acid also served to prevent depression of kidney respiration even when injected intramuscularly. This does not completely support the conclusions of the toxicity studies already outlined.

Histological examination of tissues following injections of neoarsphenamine and ascorbic acid. The animals which had been prepared for these studies were sacrificed on the third and fourth days after injection since the data on kidney respiration indicated the maximum depression on these days. The animals received 400 mgm./kgm. of neoarsphenamine intravenously with or without 100 mgm./kgm. of ascorbic acid as the sodium salt.

The pathological changes in various organs of the albino rat following injections of neoarsphenamine have been very thoroughly described by Kolmer and Lucke (16). Our pathological protocols follow:

Following the intravenous administration of nearly absolutely lethal doses of neoarsphenamine to the rat more or less marked histopathological renal changes are demonstrable. These ordinarily involve from 10 to 80% of the tubules. A typical section is shown in the accompanying photomicrograph, Plate 1A. In this section about 80% of the convoluted tubules show necrosis of the epithelial cells. The cellular nuclei are absent and the tubules appear as a non-homogenous mass of acidophilic material. The epithelium of the non-necrotic convoluted tubules presents various stages of degeneration. Certain tubules show beginning necrotic changes and others in the field contain leucocytes and detritus. Hemoglobin casts are present in many tubules together with intact erythrocytes. The interstitial tissue of the cortex is the site of polymorphonuclear leucocytic infiltration. Less severely affected kidneys show more or less numerous mitoses of tubular epithelial cells with limited swelling and vacuolation of the glomerular tufts.

The liver and spleen of these animals show few or no characteristic deviations from normal.

In the series of animals which received the same dosages of neoarsphenamine, but also received 100 mgm./kgm. of ascorbic acid no gross pathological changes were usually demonstrable. The histopathological preparations of the tissues of these animals were carefully studied. In only one of six animals were morbid changes found comparable to those described above (see Plate 1B). The epithelium of many convoluted tubules in the deeper portions of the cortex is the site of albuminous (parenchymatous) degeneration. No necrotic changes are demonstrable and mitoses are absent. A typical section from an ascorbic acid animal, free of pathology, is shown in Plate 1C.
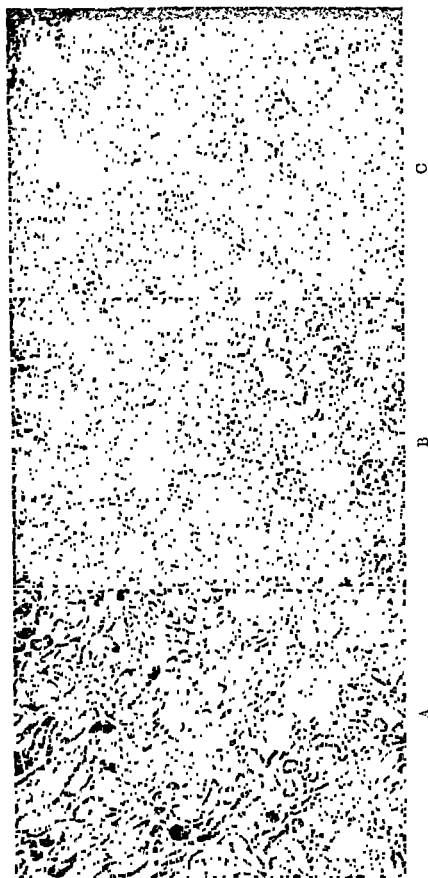
PLATE I

SECTIONS OF KIDNEY OF TREATED ANIMALS × 75

A Four days after administration of 400 mgm./kgm. of neoarsphenamine Extensive necrosis of epithelium of convoluted tubules. B Four days after administration of 400 mgm./kgm. of neoarsphenamine and 100 mgm./kgm. of ascorbic acid. Mild parenchymatous degeneration of epithelium of convoluted tubules in the deeper portions of the cortex. C. Same medication as (B) in another animal. No morbid changes

As would be expected, there was no demonstrable histopathology in the liver or spleens of these animals.

DISCUSSION. On the basis of the incidence of survival, ascorbic acid is capable of largely detoxifying neoarsphenamine administered intravenously as described in the N.I.H. regulations. Dosages of the arsenical which represent LD_{90} will kill only about 10% of the animals providing the neoarsphenamine is injected together with an equal weight of ascorbic acid as its sodium salt. However, the amount of the acid actually needed to exert this protective effect lies between one-eighth and one-fourth of the weight of the arsenical, or about 0.5 mole of ascorbic acid for 1 mole of neoarsphenamine. Still larger doses of neoarsphenamine may be detoxicated if the amount of ascorbic acid is increased beyond this. At a level of 3 moles ascorbic acid to 1 mole of neoarsphenamine, the dose of neoarsphenamine may be increased to 700 mgm./kgm. with no greater toxicity than that observed in the controls receiving 400 mgm./kgm. of neoarsphenamine. If the ascorbic acid is injected simultaneously at another site (e.g., intramuscularly), its detoxifying action is somewhat decreased but not entirely eliminated. If injected intramuscularly two hours before the neoarsphenamine, its protective action is lost. When isoascorbic or d-glucoascorbic acid is injected along with the arsenical either exerts the same protective effect as ascorbic acid itself. The action therefore, is not associated with vitamin activity and appears to be entirely a matter of the prevention of oxidation. This oxidation may occur partly due to oxygen dissolved in the solvent, but the evidence indicates that the ascorbic acid acts largely within the organism by the prevention of further oxidation, and possibly by the reversal of such slight oxidation as has already occurred. It is generally believed that neoarsphenamine is oxidized in the organism as follows: neoarsphenamine → arsine oxide → arsonic acid → inorganic oxide. The ascorbic acid may act by retarding the conversion of neoarsphenamine to arsine oxide or colloidal forms (17). We do not confirm Martin and Johnson in their finding that ascorbic acid is an effective detoxicant if given two hours before the arsenical.[3]

Of the other organic acids tested, only lactic and p-aminobenzoic acids appear to exert any protective effect. In the case of the former 3 moles are required to produce a protective effect equal to that of 0.5 mole of ascorbic acid. In the case of the latter, one mole suffices to protect against one mole of the arsenical, if they are injected intravenously in the same solution. The protective effect is somewhat diminished if different sites of injection are used.

Studies of the respiration of kidney slices from animals which have received injections of toxic dosages (400 mgm./kgm.) of neoarsphenamine reveal a marked diminution in oxygen consumption, particularly on the second to fourth days after injection. If a sufficient amount of ascorbic acid (100 mgm./kgm.) or p-aminobenzoic acid (125 mgm./kgm.) is given along with the neoarsphena-

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[3] In a study of time relationships, similar to that carried out by Sandground, we have found that the effectiveness of ascorbic acid is considerably impaired if it is injected (intravenously) more than 10 minutes before or more than 10 minutes after the neoarsphenamine.

mine, either no depression of kidney respiration is noted, or the depression is considerably less

The injection of toxic dosages of neoarsphenamine results in characteristic renal lesions, but there is little definite pathology in liver or spleen If the neoarsphenamine is injected along with a suitable quantity of ascorbic acid, no pathological changes are demonstrable by histological examination of the organs studied

The simultaneous injection of neoarsphenamine and ascorbic (or isoascorbic) acid results in no significant change in the excretion of arsenic, as compared to control animals injected with the arsenical only, nor is there any significant difference in the arsenic content of liver or kidney at various time intervals after the injection However, in the presence of neoarsphenamine the excretion rates of ascorbic and isoascorbic acids are decreased since rather large amounts of these acids are still in the blood stream after 24 hours In control animals receiving only ascorbic (or isoascorbic) acid normal blood levels have been reestablished in 24 hours in spite of the extremely high initial values

There is little to choose between ascorbic and p aminobenzoic acids as detoxicants for neoarsphenamine They are about equally effective in reducing the toxicity of the arsenical and both are in themselves practically non toxic (18, 19)

If the detoxication of neoarsphenamine by means of organic acids is to be of value, it is necessary that the chemotherapeutic effects of the former should not be appreciably altered by the detoxicants Sandground and Hamilton (8) have shown that the trypanocidal action of Carbarsone and arsanilic acid is not inhibited by p aminobenzoic acid Data available in this laboratory indicate that the trypanocidal action of Glyxarsenyl (3,4' diacetylamino 4 hydroxy arsenobenzene 2 glycolic acid) is not inhibited by the presence of 0 5 or 1% of ascorbic or isoascorbic acid in the solution nor is the trypanocidal action of neoarsphenamine inhibited by the simultaneous injection of ascorbic or isoascorbic acid at a level of 3 moles for each mole of neoarsphenamine Furthermore our experimental studies on rabbits infected with *Treponema pallidum* (Nichols strain) have shown that the inclusion of ascorbic or isoascorbic acids in Glyxarsenyl does not alter its therapeutic effectiveness

The authors are greatly indebted to the following persons for considerable technical assistance with these experiments Hugo Kocher Donald Seppelin, Erich Danziger, and Mary Fazekas

SUMMARY

The toxicity of neoarsphenamine for albino rats is materially reduced by ascorbic, isoascorbic, d glucoascorbic, and p aminobenzoic acids The most favorable effect is obtained if the arsenical and protective agent are injected intravenously in the same solution, but the acids are somewhat effective if injected simultaneously at another site The function of the ascorbic acids appears to be primarily that of preventing oxidation, chiefly after injection The mechanism by which p aminobenzoic acid reduces toxicity is obviously different and

has been discussed elsewhere (10). There is evidence that the therapeutic efficiency of some typical arsenicals is not altered by the detoxicants.

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THE PHARMACOLOGIC BASIS FOR THE WIDELY VARYING TOXICITY OF ARSENICALS

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Although arsenicals have proved effective in the treatment of a wide variety of spirochetal and protozoal diseases, the fundamental mechanism of their parasiticidal action, or of their toxic effect on the host, is not wholly clear   In a classic discussion, Ehrlich (1) presented the thesis that *"corpora non agunt nisi fixata"*, that chemotherapeutic agents in general can exert their therapeutic effect only if bound by the parasite, and that their toxic action is due to a similar combination with vital tissues of the host   More specifically, evidence has been presented by Voegtlin, Dyer and Leonard (2) and by Rosenthal (3) that organic arsenicals owe their activity to their combination with physiologically essential sulfhydryl groups in the host tissues or in the parasite

It is a possible corollary both of Ehrlich's general thesis and of the sulfhydryl theory, that arsenicals may be active only to the degree that they are bound, and that the enormous differences in the toxicity of arsenic compounds may be due merely to their varying affinity for the body tissues   Suggestive evidence in this direction has been presented by Thuret (4)   Trivalent phenyl arsenoxides are known to be many times more toxic than the corresponding pentavalent arsonic acids, and Thuret found that the trivalent compounds were bound by a suspension of red blood cells in vitro, while the pentavalent compounds were not

The present paper offers further evidence that the toxicity of arsenic compounds is quantitatively a function of their affinity for tissue   A large series of phenyl arsenoxides (5), arsphenamines and arsonic acids of widely varying toxicity has been studied with respect to binding by red blood cells in vitro   In a smaller series of compounds, the degree of binding by red blood cells and by tissues after intravenous injection, and the rate of excretion, have also been studied   In general, the toxicity of the compounds has been so closely correlated with the degree to which they are bound by the tissues, and thus with the rate of excretion, as to make a causal relationship highly probable   Moreover, the binding of phenyl arsenoxides by red blood cells in vitro has proved to be a model for their combination with tissues in vivo, and has so closely paralleled their systemic toxicity as to provide a reliable measure of that factor   The degree to which these generalizations must be modified by the in vivo conversion of some arsenicals to other more toxic compounds is discussed in the text

The present findings are thus in complete accord with the concepts of parasito tropism and organotropism postulated by Ehrlich (1)   The varying toxicity

[1] With the technical assistance of Emily B Watson

of arsenicals is a function of their organotropism, i.e., affinity for tissues; and there is reason to believe that in this same series of compounds, the therapeutic activity is similarly a function of parasitotropism, i.e., the amount of arsenic bound by the pathogen. Under such circumstances, the chemotherapeutic index of an arsenical (the ratio between the maximum tolerated dose and the minimum curative dose) may be simply an expression of the relative affinity of that compound, or of an in vivo derivative, for the tissues of the host and for the invading organism.

METHODS AND MATERIALS. *The binding of arsenic compounds by red blood cells in vitro.* As a result of preliminary tests with respect to the effect of the concentration of red blood cells and of arsenic on the rate and degree of arsenic combination, the following standardized technic was used in the *in vitro* experiments described in the present paper.

Red blood cells were obtained immediately before each experiment by bleeding two or more normal rabbits into potassium oxalate (approximately 2 to 5 mg. of dry oxalate, or 0.1 cc. of a 5 per cent solution, per cc. blood). The oxalated blood was then diluted with seven volumes of normal oxalated plasma. No attempt was made to correct for the minor variations in the hematocrit readings of individual rabbits. Two cc. of arsenical solution containing a total of 200 micrograms of arsenic were added to 20 cc. of the blood dilution, giving a final arsenic concentration of 9.09 micrograms of arsenic per cc. Five cc. aliquots were centrifuged (in duplicate) after 10 and 120 minutes. In the interim the mixture had been inverted at frequent intervals to prevent settling out of cells. The plasma was removed immediately following centrifugation and the arsenic content of both cells and supernatant plasma determined.

In vivo studies. Rabbits were injected intravenously with 0.0033 millimols (0.25 mg. arsenic) of the various arsenic compounds per kg. body weight. This relatively small dosage was selected in order to study the distribution of the arsenicals in vivo under physiological conditions, and not in tissue so altered by the toxic effects of the drug as to result ultimately in the death of the animal. With most compounds these small doses did not cause the temporary suspension of urine and stool excretion sometimes observed after sublethal doses of toxic arsenicals. Moreover, the dose selected is approximately the dosage of mapharsen used in clinical practice, and is below the lethal dose of the highly toxic parent compound, the unsubstituted phenylarsenoxide. The arsenic solutions used were carefully adjusted to pH 6.8–7.2 before injection. The arsenic concentrations in whole blood, red blood cells, and plasma were determined at intervals after injection, as well as the amounts excreted in the urine and stool. At varying periods the animals were sacrificed and the arsenic content of the tissues determined.

For the blood arsenic levels, the rabbits were bled from the heart into dry oxalate at varying intervals after the injection of the drug. Aliquots were centrifuged immediately (within 1–2 minutes), the plasma removed, and the arsenic concentration in the whole blood, red blood cells and plasma determined.

Arsenic analysis. The method of arsenic analysis used was essentially that described by Chaney and Magnuson (6) with the following minor modifications. Reagents: The reducing mixture contained 8 per cent KBr instead of 4 per cent as specified by Chaney and Magnuson, because better recovery of known samples was obtained at the higher concentration. The concentration of the hydrazine solution used to develop color was increased from 0.15 per cent to 0.3 per cent. A zero blank (against distilled H_2O) could then be regularly obtained.

Procedure. All tissues, urine and stool specimens were digested and distilled twice to minimize contamination with phosphorus. In concentrating the first distillate by boiling, preparatory to re-distillation, it was found necessary to add about 3 cc. nitric acid before the sulfuric acid in order to avoid significant losses of arsenic.

The effect of the concentration of arsenic and of red blood cells on the amount of arsenic bound by red blood cells in vitro The effect of the concentration of arsenical on the amount of arsenic bound by red blood cells is illustrated in figure 1, in which 3 NH_2-4-OH phenyl arsenoxide was the test compound Although the percentage of arsenic bound by red blood cells decreased at the higher concentrations, the absolute amount bound increased continuously up to the highest concentration studied, at which there were 1470 micrograms of arsenic per cc red blood cells Even at this high level, at which damage to the red blood cells was evidenced by moderate hemolysis, the curve offers no evidence that the red blood cells were saturated with respect to the arsenoxide
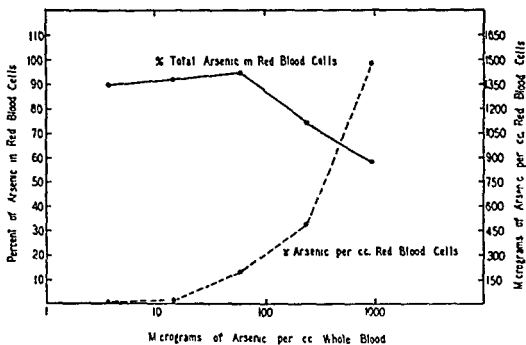

FIG 1 THE EFFECT OF THE CONCENTRATION OF 3 NH_2 4 OH PHENYL ARSENOXIDE ON THE AMOUNT OF ARSENIC BOUND BY RED BLOOD CELLS IN VITRO

The rate at which arsenic was bound by the red blood cells is illustrated in table 1 and figure 2 Under the conditions of the experiment, phenylarsenoxide was bound rapidly and almost completely (86 7 per cent in 5 minutes) while its relatively non toxic 3 NH_2 4 OH derivative was only gradually bound

As is apparent from figure 2, differences in the rate of combination of the various arsenicals with red blood cells which were apparent on short exposure (10 minutes), might no longer be apparent after the reaction had been allowed to continue for two hours Thus, in five minutes the red blood cells in whole blood had bound 3 5 times as much of the phenyl arsenoxide as of the 3 NH_2-4 OH derivative, but after two hours there was no significant difference

The effect of the concentration of red blood cells on the rate of arsenic binding is summarized in figure 3 As there shown, differences in affinity for the arseni

TABLE 1

The rate of combination of arsenical by red blood cells

To whole blood containing 36 per cent red blood cells was added 1/10 volume of arsenical solution containing 100 micrograms of arsenic per cc., giving a final concentration of 9.1 micrograms of arsenic per cc., and 33 per cent red blood cells, in the reacting mixture.

COMPOUND USED	ARSENIC CONCENTRATION (MICROGRAMS/CC.) AFTER					REMARKS
	5'	15'	30'	60'	240'	
C_6H_5AsO						
Plasma................	0.57	0.55	0.5	0.38	0.5	Rapid combination
Red blood cells...........	23.9	23.9	23.9	23.9	25.6	of the toxic com-
Per cent of total arsenic in						pound by red.
red blood cells*.........	86.7	86.7	86.7	86.7	93.0	blood cells
$3\text{-}NH_2\text{-}4\text{-}OH\text{-}C_6H_3AsO$ (Mo-						
lar toxicity 7.9 per cent						
that of the above unsub-						
stituted compound)						
Plasma................ ...	14	10.2	7.2	4.3	0.1	Non-toxic compound
Red blood cells....... ...	6.83	12	15.5	20.3	25.1	bound by red.
Per cent of total arsenic in						blood cells only
red blood cells....... ..	24.6	43.5	56.3	73.7	91.3	slowly

$$* \frac{0.33 \times \text{As content of red blood cells}}{9.1} \times 100$$
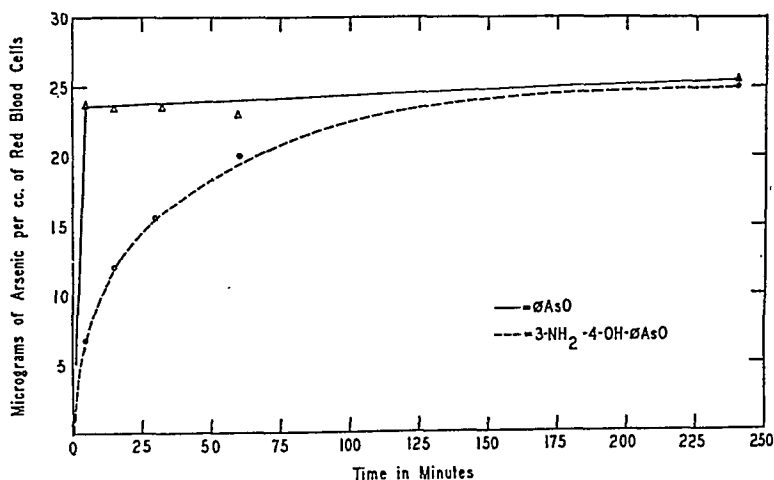
FIG. 2. THE RATE OF COMBINATION OF PHENYL ARSENOXIDES BY RED BLOOD CELLS

To whole blood containing 36 per cent red blood cells was added $\frac{1}{10}$ volume of arsenical solution containing 100 micrograms of arsenic per cc., giving a final concentration of 9.1 micrograms of arsenic per cc., and 33 per cent red blood cells, in the reacting mixture. In this and all following figures, the symbol ϕ is used for the phenyl ring.

cals were accentuated by decreasing the concentration of red blood cells used. In that experiment, a 1:8 dilution of blood bound five times as much phenyl arsenoxide as it did of the 3-NH₂-4-OH derivative; but the ratio was only 1 02 when whole blood was used.
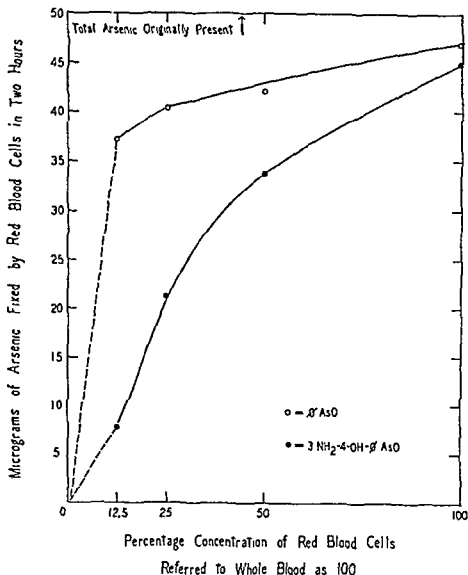
FIG 3 THE EFFECT OF THE CONCENTRATION OF RED BLOOD CELLS ON THE AMOUNT OF ARSENIC BOUND

In order to make manifest differences in the affinity of red blood cells for the various arsenic compounds, it was therefore necessary to minimize the concentration of the arsenical and of the red blood cells, and to minimize also the time for which the red blood cells were exposed to the arsenic solution   Accordingly, in the following experiments, whole blood was diluted 1:8 with normal plasma

prior to use, one volume of a solution containing 100 micrograms of arsenic per cc. was added to 10 volumes of the cell suspension, and the amounts of arsenic bound by the cells were determined after both 10 and 120 minutes.

The varying affinity of red blood cells for arsenoxides, arsphenamines and arsonic acids in vitro. With this standardized technic, the results shown in table 2 were obtained. As tabulated in the first section of that table, and graphically summarized in figure 4, the amounts of the various phenyl arsenoxides (acid-substituted compounds excepted) bound by red blood cells in vitro, whether in 10 minutes or 120 minutes, and the ratio of the arsenic concentrations in the cells and in the plasma, agreed to a striking degree with their systemic toxicity in white mice. In this series of compounds, the amount of an arsenoxide bound by the cells in vitro was a reliable index of its systemic toxicity. This strongly suggests that a suspension of red blood cells is a valid model for the body tissues, and that the toxicity of phenyl arsenoxides is primarily a function of the degree to which they are bound by tissues.

Three other groups of compounds are listed in table 2, which are not included in figure 4. The first is a series of *arsphenamines*. Some of these are known to oxidize in solution to a considerable degree, and there is good reason to believe that their treponemicidal activity and toxicity rest on a similar oxidation in vivo (7, 8, 9). Although the amount of arsenic bound by red blood cells from arsphenamine solutions in vitro roughly paralleled their toxicity in vivo, both values probably reflect the degree to which the compounds had been oxidized to the corresponding arsenoxides. Accordingly, not much significance is to be attached to the data with respect to the binding of these compounds by red blood cells in vitro.

Thuret's observation that *arsonic acids* are bound only to a slight degree by red blood cells in vitro has been amply confirmed (third section of table 2). The minimal degree of arsenic binding is paralleled by the relative non-toxicity of these compounds in vivo. This correlation is, however, true only in a general sense. For individual compounds there was not the quantitative correlation between these two factors noted in the case of the phenyl arsenoxides. As shown in table 2, all the arsonic acids were bound by red blood cells to approximately the same, and slight, degree; but the systemic toxicity of these compounds varied between wide limits. Ehrlich (10) and Roehl (14) suggested that pentavalent arsenicals are reduced in vivo to the corresponding and far more toxic trivalent arsenoxide. Voegtlin, Dyer and Leonard (2), Voegtlin, Smith and Crane (8), Lourie, Murgatroyd and Yorke (10a), and Thuret (11), with a varying experimental approach, have come to the same conclusion. Under these circumstances, one would not anticipate a correlation between the binding of arsonic acids by red blood cells in vitro, and their systemic toxicity.

The third group of compounds in table 2 not represented in figure 4 is a series of *acid-substituted phenyl arsenoxides.* These were regularly far more toxic than would have been anticipated from the minimal degree to which they were bound by red blood cells in vitro. There is reason to believe that this dis-

TABLE 2

The correlation between the systemic toxicity of arsenic compounds and their affinity for red blood cells in vitro

To whole blood diluted with 7 volumes of normal plasma (1 8) was added $\frac{1}{10}$ volume of arsenical solution containing 100 micrograms As/cc giving a final concentration of 9 1 micrograms As/cc Aliquot portions were centrifuged after 10 and 120 minutes at room temperature, and the arsenic content of the red blood cells determined

TYPE OF COMPOUND	SPECIFIC COMPOUND	PARTITION OF ARSENIC BETWEEN RED BLOOD CELLS AND PLASMA AFTER						MOLAR TOXICITY REFERRED TO THAT OF PHENYL ARSEN OXIDE AS 100
		10			120			
		γ As/cc		RBC As Plasma As Ratio	γ As/cc		RBC As Plasma As Ratio	
		RBC	Plasma		RBC	Plasma		
Phenyl arsenoxides	4 SO₂NH₂	7 5	8 75	0 9	17	8 3	2 1	4 8
	4 CONHCH₂CONH₂	8 9	8 55	1 2	22 3	8 3	2 7	5 5
	3 NH-4 OH	10 2	8 0	1 3	38	7 9	4 8	7 9
	4 CONH₂	9 1	8 9	1 0	42 2	7 7	5 5	9 6
	4 CH=CHCONH₂	13	7 65	1 7	47 5	6 0	7 9	10 0
	3 OH-4 CONH₂	20 8	7 0	2 9	89 4	5 9	15	23
	4 SO₂NHC₂H₅	34 8	8 0	4 5	126	3 5	36	32
	3,4 ⟨N-O⟩CNH₂	48	7 9	6 2	77	3 2	24	37
	3 NH₂-4 C₂H₄OH	96	5 1	18 9	150	2 5	61	44
	4 NH₂	129	2 8	46	145	—		56
	2 OH 3 NH₂	128	3 0	43	120	2 8	43	78
	2 NH₂	183	2 8	67	184	2 6	73	84
	4 N(CH₃)₂	155	2 5	62	134	2 9	46	86
	Unsubstituted phenyl arsenoxide	180	1 9	94	180	2 1	87 5	100
Arsphenamines	Neoarsphenamine	4	8 8	0 5	8	9	0 9	1 4
	Sulpharsphenamine	4 7	8 95	0 5				
	Arsphenamine	4 2	8 8	0 5	2 1	7 8	0 3	2 0
	Silver arsphenamine	12	10	1 2	15 5	9 15	1 7	3 0
Arsonic acids	4 NHCH₂CONH₂	3 0	8 3	0 4	3 7	9	0 4	0 1
	4 OH	2 1	7 8	0 3	3 2	6 8	0 5	0 2
	3 COOH	4 5	9 2	0 48	4 5	9 65	0 47	0 16
	3 NH₂ 4 OH	0 5	7 8	0 64	3 5	7 8	0 45	0 14
	4 N(CH₃)₂	1	8 8	0 11	1	8 3	0 12	
	Unsubstituted phenylarsonic acid	4 1		0 24		9 2	0 54	5 55
Acid substituted phenyl arsenoxides	4 (CH₂)₂COOH	4 5	8 9	0 51	4 4	8 6	0 5	8 8
	4 CH=CHCOOH	4 5	8 4	0 54	5 0	8 6	0 6	9 35
	3 COOH	4 75	10	0 48	7 5	9 25	0 8	16
	4 SO₂Na	4 25	10 4	0 4	4 7	10 3	0 5	20
	4 COOH	4 75	10	0 5	4 5	10 1	0 4	41
Inorganic arsenicals	NaAs(OH)₂	10	7 2	1 4	14 5	7 5	1 9	8
	Na₂HAsO₄ 7H₂O	1	8 6	0 1	1 5	8 7	0 2	3

crepancy is due to the fact that acid-substituted phenylarsenoxides are rapidly converted in the body to compounds with a greater affinity for tissue, and which are correspondingly more toxic. Two distinct lines of evidence point to that conclusion.

The first is the rate of excretion of acid-substituted arsenoxides after their intravenous injection. As will be discussed in greater detail in a following section, there is a high inverse correlation between the toxicity of phenyl arsenoxides, and the rate at which they are excreted, i.e., the less toxic the compound, the faster it is excreted. Thus, the 3-NH_2-4-OH compound (mapharsen), one of the least toxic of the arsenoxides tested, was excreted fairly rapidly, approximately 15 per cent appearing in the urine in 4 hours and 55 to 85 per cent appearing in the urine and stool within 48 hours after the intravenous injection of doses (0.86 mg./kg.) approximating those used in man. On this basis, the more toxic 3-NH_2-4-COOH, 4-COOH, 4-CH_2COOH, or 4-$(CH_2)_3$COOH compound should have been excreted more slowly than mapharsen. Instead, all four of these acid-substituted arsenoxides were at first excreted rapidly, as much as 40 per cent appearing in the urine within 1 hour. However, after that initial extremely rapid excretion, relatively small amounts were eliminated during the following 4 days. By that time, mapharsen had been excreted almost quantitatively (cf. fig. 5). A logical explanation is that these acid-substituted arsenoxides are converted in vivo to other compounds which are excreted only slowly, and which are presumably also more toxic than the original compound. On this basis, the initial rapid excretion is that of the unchanged acid compound, with a known low affinity for red blood cells in vitro, a low affinity for body tissues in vivo, and with a correspondingly low toxicity. When the acid phenyl arsenoxide is presumably converted to this as yet unidentified and toxic derivative, its excretion is abruptly curtailed.

The second point which supports the thesis that acid-substituted arsenoxides are converted in vivo to other, more toxic, derivatives, is the rate at which mice die after their parenteral administration. After intraperitoneal injection at the LD_{50} level, the toxic effects of most phenyl arsenoxides were acute, more than 60 per cent of the total observed deaths occurring in the first 24 hours. With similar doses of acid-substituted compounds, death was characteristically and regularly delayed, with the peak mortality only 48 hours after injection, and with a considerable proportion of the animals dying 72 and 96 hours after injection (cf. fig. 6).

The initially rapid excretion of acid-substituted arsenoxides in rabbits, the sudden decline in the rate of that excretion after the first few hours, and their delayed toxicity in mice, strongly suggest that, as indicated by the minute amounts bound by red blood cells in vitro, these acid-substituted compounds are relatively non-toxic as such, and that their unexpectedly high toxicity in vivo is due to their conversion to other compounds.

To recapitulate, in the case of arsphenamines, arsonic acids, and acid-substituted phenyl arsenoxides, their known or probable conversion in vivo to more toxic compounds largely vitiates the significance of their affinity for red blood

cells in vitro as a measure of systemic toxicity. For the other arsenoxides listed in the first section of table 2, and presumably for any arsenical which is not converted to more toxic compounds in vivo, the amount bound by red
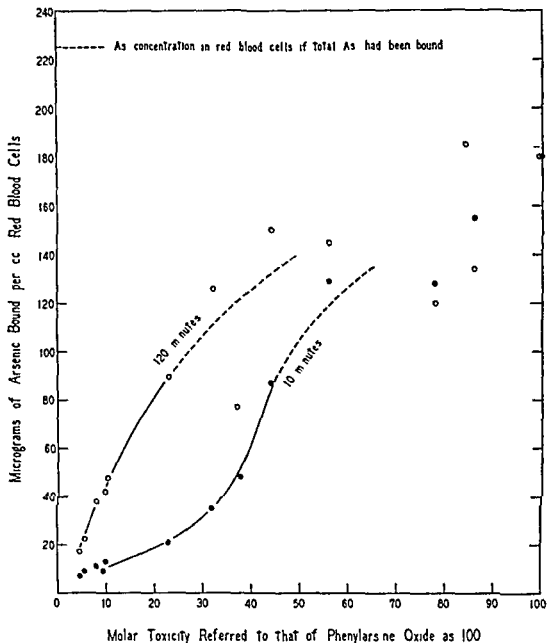

FIG 4 THE CORRELATION BETWEEN THE SYSTEMIC TOXICITY OF PHENYL ARSENOXIDES AND THEIR AFFINITY FOR RED BLOOD CELLS IN VITRO (BASED ON DATA OF TABLE 2)

One cc of the various arsenical solutions each containing 100 micrograms of arsenic, was added in duplicate to 10 cc of a 1 8 dilution of rabbit blood in normal rabbit plasma (final concentration = 9 1 micrograms of arsenic per cc). After standing for 10 minutes and for 120 minutes at room temperature duplicate 5 cc portions of each tube were centrifuged and the arsenic content of the cells and plasma determined. The volume of sedimented red blood cells in each such 5 cc portion averaged 0 2 cc.

blood cells is apparently a reliable criterion of relative toxicity. Sodium arsenite and sodium arsenate are cases in point. Their relative molar toxicities in mice were 8 1 and 3 1 respectively (referred to that of phenylarsenoxide as 100, LD$_{50}$
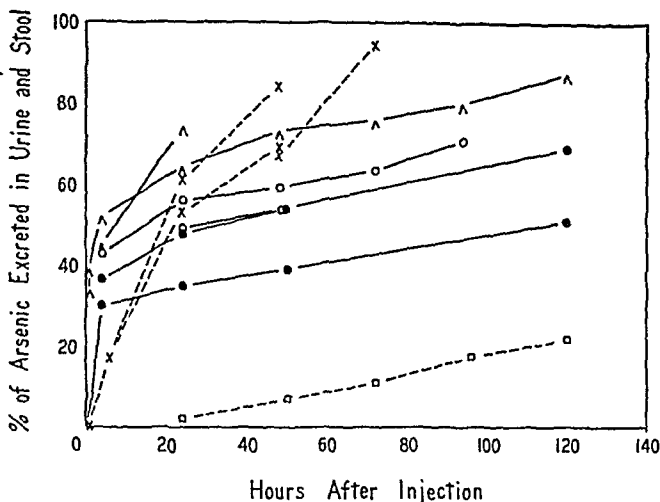
FIG. 5. THE RATE OF ARSENIC EXCRETION IN URINE AND STOOL FOLLOWING THE INTRA-VENOUS INJECTION OF ACID-SUBSTITUTED PHENYL ARSENOXIDES (SOLID LINES), COMPARED WITH THAT OF MAPHARSEN (x----x) AND PHENYL ARSENOXIDE (□----□).

Rabbits were injected with equimolar amounts (0.0033 millimols per kg.) of the various arsenicals in neutral solution. Urine was obtained by catheterization at intervals thereafter. The points indicated in the figure represent the cumulative total arsenic excretion in the urine and stool, and in the case of all the 1 and 4-hour analyses represent urine excretion only.

x----x, 3-NH$_2$-4-OH-; O——O, 4-COOH-; ●——●, 4-CH$_2$COOH-; ∧——∧, 4-(CH$_2$)$_3$COOH-; □----□, Unsubstituted phenyl arsenoxide.
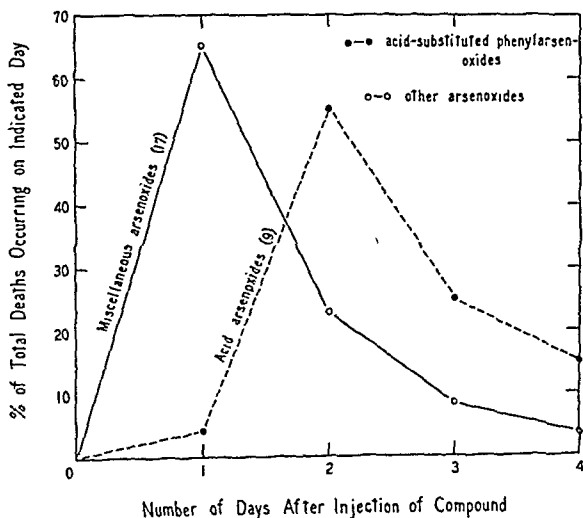

FIG. 6. THE RATE OF DEATH OF MICE INJECTED INTRAPERITONEALLY WITH 80 TO 120 PER CENT OF THE LD$_{50}$ AMOUNTS OF VARIOUS PHENYL ARSENOXIDES

●----●, composite of 9 acid-substituted compounds (150 dead; 159 survived). O——O. composite of 7 amide-substituted and 10 miscellaneous phenyl arsenoxides (258 dead: 244 survived).

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values 19 and 114 mg/kg, respectively) Corresponding to that three fold difference in molar toxicity, there was a ten fold difference in the amount of arsenic bound by red blood cells in vitro (bottom of table 2)

These data indicate that the binding of arsenic by red blood cells in vitro may be a model for their binding by tissues in vivo, and that, in general, the widely varying systemic toxicity of arsenicals may reflect only the varying degree to which they, or their in vivo derivatives, are bound by tissues

The arsenic content of the red blood cells and plasma at varying intervals after the intravenous injection of arsenicals, in relation to toxicity   Tables 3 and 4 show

TABLE 3

Concentration of arsenic in red blood cells and plasma at varying intervals after the intravenous injection of arsenicals (0 0033 millimols/kg = 0 25 mg As/kg)

COMPOUND	RABBIT NUMBER	AS IN WHOLE BLOOD (γ/cc) AFTER			AS IN RED BLOOD CELLS (γ/cc) AFTER			AS IN PLASMA (γ/cc) AFTER		
		1±	10	30	1±	10	30	1±	10	30
C_6H_5AsO (Unsubstituted phenyl arsen oxide)	6172	4 5	1 9	0 8	16 8	6 7	1 8	0 16	0 05	0 025
	7549			0 6			1 8			0 12
	7592	3 1	1 5		9 8	6 4		0 3	0 17	
	8290	3 5			9 57			0 13		
3 NH_2-4 C_6H_4OH C_6H_4AsO	6629	3 2			7 7			0 5		
	6706	3 4		0 9	7 2		2 4	0 4		0 17
3 - NH_2 4 OH $C_6H_3AsO_3$	1	0 7						0 95		
	6146	1 2		0 5	0 86			1 27		0 53
	6299	1 0		0 15			0 49	1 42		0 18
	7458	1 37			1 54±			1 29		
	7544			0 13			0 28			0 06
	8568	0 87(?)			1 23			1 16		
	6291		0 55			0 42			0 25	
Tryparsamide (4 $NHCH_2CONH_2$ $C_6H_4AsO_3H_2$)	1	1 2		0 35	0 08		0 06	2 37		0 47
	7588	1 48		0 53			0 08	2 2		0 74
	8577	1 57						2 0+		

the concentration of arsenic in the whole blood, red blood cells and plasma at varying intervals after the intravenous injection in equimolar amounts, of four arsenicals of widely varying toxicity With all four of these compounds, only minute concentrations of arsenic (0 1–0 2 micrograms per cc) remained in the blood after 2 hours If 7 per cent of the total body weight is taken as the approximate blood volume this represents less than 6 per cent of the arsenic originally injected There was, however, a marked difference in the rate at which the various compounds disappeared from the blood, and in their distribution between red blood cells and plasma In the case of the most toxic compound the unsubstituted phenyl arsenoxide, essentially all of the injected arsenical was still in the blood stream ¾ to 1¹ minutes after injection and more

than 99 per cent was in the red blood cells. In that same time interval, however, more than half of the relatively non-toxic tryparsamide had left the blood stream, and of the remainder, more than 95 per cent was in the plasma.

Comparing the four compounds tested, with relative molar toxicities of 100, 44, 7.9 and 0.09, the percentage of the circulating blood arsenic in the red blood cells approximately 1 minute after injection was > 95, 75–95, 25–75 and 5 re-

TABLE 4

The partition of arsenic between red blood cells and plasma, considered in relation to the toxicity of the compound (calculated from experimental data of table 3)

COMPOUND	RABBIT NUMBER	AVERAGE % OF INJECTED ARSENIC REMAINING IN CIRCULATING BLOOD AFTER			AVERAGE % OF TOTAL BLOOD ARSENIC IN RED BLOOD CELLS*	RATIO OF RBC As:PLASMA As				RELATIVE MOLAR TOXICITY OF COMPOUND†
						After			Average value	
		1'±	10'	30'		1'±	10'	30'		
C_6H_5AsO (Unsubstituted phenyl arsenoxide)	6172	>95	53	22	95–99	100	125	78	65±	100
	7549			16				15		
	7592	80	42			30	40			
	8290	>95								
$3\text{-}NH_2\text{-}4\text{-}C_2H_4OH\text{-}C_6H_3AsO$	6629	88			75–95	17			15	44
	6706	>95		25		18		14		
$3\text{-}NH_2\text{-}4\text{-}OH\text{-}C_6H_3AsO$	1	20			25–75				1.5	7.9
	6146	34		14		0.7		0.9		
	6299	28		4						
	7458	25								
	7544			4		1.2				
	8568	24				1.0		5.0		
	6291		15				1.7			
Tryparsamide (4-$NHCH_2CONH_2\text{-}C_6H_4AsO_3H_2$)	Y	34		10	5±	0.03		0.13	0.09	0.09
	7588	42		15				0.11		
	8577	44								

* $\dfrac{\text{Red blood cell As concentration} \times \text{percentage by volume of red blood cells in blood}}{\text{As concentration in whole blood}}$: no significant differences noted between 1', 10' and 30' intervals.

† On intraperitoneal injection into white mice, referred to that of phenyl arsenoxide as 100.

spectively, and the ratios of $\dfrac{\text{arsenic concentration in red blood cells}}{\text{arsenic concentration in plasma}}$ averaged 65, 15, 1.5 and 0.09, respectively.

The course of events in the circulating blood after the intravenous injection of phenyl arsenoxides is therefore consistent with previous observations with respect to their binding by red blood cells in vitro. The more toxic the compound, the larger was the proportion of the circulating arsenic in the red blood cells. Probably in consequence of that fact, the toxic compounds left the blood stream at a somewhat slower rate than the non-toxic compounds; but

in the case of all the compounds tested, relatively small amounts remained in the circulating blood after two hours

The correlation between the systemic toxicity of arsenicals and the degree to which they are bound by tissues in vivo   In the preceding sections, it has been shown that there is a high degree of correlation between the toxicity of phenyl arsen

TABLE 5

The correlation between the toxicity of various arsenicals, and the arsenic concentration in the liver and kidney 48 hours after their injection in equimolar amounts (0 0033 millimols/kg = 0 25 mg As/kg)

COMPOUND	RELATIVE TOXICITY REFERRED TO THAT OF PHENYLAR SENOXIDE AS 100*	RABBIT NUMBER	ARSENIC CONTENT 48 HOURS AFTER INJECTION OF			
			Liver		Kidney	
			Total γ	Micrograms/100 gm	Total γ	Micrograms/100 gm
Phenyl arsenoxides						
Unsubstituted phenyl ar senoxide	100	6274	68	90	11	50
		8521	202	260	21 5	150
4 N(CH₃)₂	87	8794	86	95	8 9	41
		8798	111	123	11 5	65
4 NH₂	56	6819	51	50	5±	25
		9077	9 7	10	4 1	40
3 NHCONH 4	37	7162	47	44	10	50
		9204	6 7	9	4 5	30
4 NHCOCH₃	21	9181	12 9	8	3 5	18
		9332	9 4	8	2 7	17
4 CH₂NH₂	13	8150	4 4	9 5	8 5	53
		8184	7 5	9	7 7	39
3 NH₂ 4 OH	7 9	N B	2 4		1 5	7±
		5971	2±	1 7	0 6±	3
		6154	1 4	2 0	3 1	17
Arsonic acid						
Tryparsamide	0 09	6466	2±	3	1 6±	8
		6821	2	2	1	5

* Based on intraperitoneal injection into white mice, essentially similarly relative toxicity observed in rabbits in the five compounds tested

oxides and their affinity for red blood cells, whether in the test tube or in the circulating blood   Highly toxic compounds were found to be rapidly and almost completely bound by the cells, while relatively non toxic compounds remained free in the plasma   As shown in table 5, these findings were duplicated in the liver and kidney   After leaving the blood stream, the several compounds were bound by these organs to varying degrees, but again in proportion to their toxicity   Thus 48 hours after the injection of various arsenicals in equi

molar amounts, the arsenic concentration in the liver was 50 to 100 times greater in the case of the unsubstituted phenyl arsenoxide than with the relatively non-toxic 3-NH_2-4-OH derivative, or the pentavalent compound tryparsamide; and, with the expected individual variations between animals, compounds of intermediate toxicity resulted in a correspondingly intermediate tissue arsenic level. It is particularly to be emphasized that these differences are probably not referable to a toxic effect of the compounds. With the exception of the unsubstituted phenyl arsenoxide, the amounts injected were well below the maximal tolerated dose, and did not cause even a temporary suspension of urine and stool excretion. It follows that the widely varying arsenic contents of the liver and kidney after the injection of the several compounds in equimolar dosage per kilogram probably reflect the varying tissue affinity of these compounds, uncomplicated by their toxic effects on these organs.

If the thesis is correct that the toxicity of arsenicals is in proportion to, and probably determined by, the degree to which they are taken up by the tissues, then no matter what arsenic compounds are used, amounts with equivalent toxic effects should result in roughly equivalent tissue arsenic levels. Thus, the LD_{50} levels of tryparsamide, phenyl arsonic acid, and unsubstituted phenyl arsenoxide, on intravenous injection into rabbits, have been found to be 700, 16, and 0.8 mg. per kg. respectively. After the injection of these compounds at the LD_{50} level, and despite the 900-fold difference in dosage, one should find comparable amounts of arsenic in the tissues. As shown in table 6 and figure 7, this has been found to be the case. The amounts of arsenic remaining in the liver, kidney, and other organs, 24 or 48 hours after the injection of 700 mg. per kg. of tryparsamide, were of the same order of magnitude as those observed after the injection of 16 mg. per kg. phenylarsonic acid, or 0.8 mg. per kg. of phenyl arsenoxide. Once the tissue levels had stabilized after the initial rapid excretion, they were comparable in amount, this despite the wide differences in the amounts of arsenic injected, and the expected variations between individual rabbits.

An apparent exception is again afforded by the acid-substituted 4-COOH phenyl arsenoxide, in that the tissue arsenic levels after the injection of an LD_{50} dose were regularly lower than with the three other arsenicals discussed in the preceding paragraph and listed in table 6. The rapid initial excretion of this compound (cf. fig. 5) is reflected in the extraordinarily high kidney levels 1 hour and 4 hours after its injection, which amounted to *19* and *11* per cent of the total arsenic injected. The low tissue levels 24 and 48 hours later were probably due to this rapid excretion; and the surprisingly high toxicity of the compound may perhaps be referable to the kidney damage caused in the first few hours after injection by a chemically modified product formed locally, in the kidney itself.

For the other three compounds studied, it is apparently the amount of arsenic firmly bound by tissue which determines the toxicity of the compound. It is interesting to note that, as will be shown in a later paper, the removal of that bound arsenic from the tissues by an appropriate detoxifying agent often prevents the death of the animal.

TABLE 6

...t the l D_{50} level of various arsenicals, and regardless of the absolute amount of arsenic injected, the tissue arsenic levels 24 to 48 hours after injection have been of the same order of magnitude*†

Figures in body of table represent arsenic concentration in parts per million of wet tissue (gamma per gram)

	1 hour				4 hours				24 hours				48 hours			
	φ AsO₂	COOH+φ AsO	φ AsOH₂	Trypasamide	φ AsO	COOH+φ AsO	φ AsOH₂	Trypasamide	φ AsO	COOH+φ AsO	φ AsOH₂	Trypasam de	φ AsO	COOH+φ AsO	φ AsOH₂	Trypasam de
Muscle	0 15	0 65	0 60	13 8		0 24		14 9	0 26	0 05	0 23	0 14	2 62	0 06	0 18	0 12
Liver	2 0, 0 2 15	0 26	4 8	4 8		0 39		70	1 92	0 18	2 7	1 11	1 5	0 16	2 8	0 86
Kidney	1 6 1 4 4	27	21 2	50		12		2 8	1 1	0 37	2 38	1 2	0 57	0 27	1 64	2 52
Brain	0 16		1 0	26 6					0 17	0 07	0 16	0 3		0 1	0 23	0 3
Blood	0 55			4 15						0 11	0 125			0 12	0 22	
Heart	1 2	0 4	3 14	7 4		0 33		0 61	0 36	0 09	0 15	0 3		0 12	0 38	0 12
Lung			3 0													
Bone	0 3	0 8		26					1 6		1 68	0 27	0 59	0 18	1 68	0 2
Skin		0 56				0 33		15 3	0 76	0 14	0 19	0 98	1 54	0 04		

* Except for the acid substituted 4 COOH phenylarsenoxide (cf text)
† Each vertical column necessarily represents an individual rabbit The amounts of the various compounds injected were as follows

	mg As/kg	mg As/kg = γ As/gm
Phenylarsenoxide	0 8	0 36
p COOH phenylarsenoxide	2 8	0 87
Phenylarsonic acid	16	5 94
Tryparsamide	700	175

†φ = phenyl ring

The rate of excretion of various arsenicals in urine and stool in relation to their toxicity. As could be predicted from the foregoing experiments, the rate of excretion of arsenic compounds was also correlated with their toxicity. The non-toxic compounds, which did not adhere to the tissues, were excreted rapidly; while the toxic compounds, firmly held in the tissues, were excreted at a correspondingly slower rate. The rates of excretion of a series of representative compounds injected in equimolar amounts (0.0033 millimols per kg. = 0.25
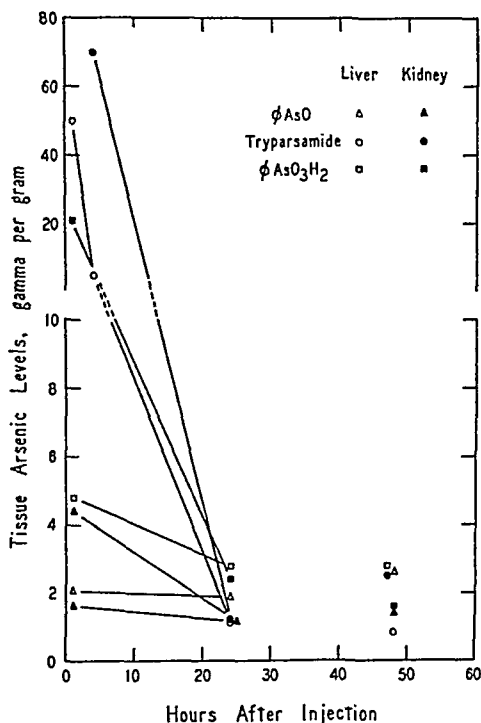

Fig. 7. Showing that the liver and kidney arsenic contents 24 and 48 hours after the injection of various arsenicals at the LD_{50} dose were essentially the same, despite a 500-fold difference in their LD_{50} level.

mg. As per kg.) are shown in table 7, and the correlation between the rate of excretion and the systemic toxicity of these compounds is clearly apparent.

As has been discussed in a preceding section, the acid-substituted phenyl arsenoxides form a special group, in that a rapid initial rate of excretion is followed by a period of slow excretion, probably due to their conversion in vivo to other, more toxic, compounds (cf. fig. 5).

DISCUSSION. The experimental data here presented strongly support the thesis that the toxicity of arsenicals is primarily a function of the degree to

which they are bound by tissues It has been shown for a large series of phenyl arsenoxides that they are bound by red blood cells in vitro in proportion to their toxicity It has been similarly found that in the case of highly toxic compounds, most of the injected arsenical had been bound by the red blood cells immediately

TABLE 7

The rate of excretion of arsenic in the urine and stool of rabbits after the injection of various arsenicals in equimolar amounts (0 0033 millimols/kg = 0 25 mg As/kg), considered in relation to the toxicity of the compound

COMPOUND	ANIMAL NUMBER	CUMULATIVE PERCENTAGE OF As EXCRETED IN DAYS							RELATIVE MOLAR TOXICITY*	AVERAGE % EXCRETED IN	
		1	2	3	4	5	6	7		24 hr	48 hr
C₆H₅AsO	N B 1	2 5	8	12	17 4	22		35 4	100	5	7
	6274	7 5	9 4								
	6460		3 5	5 0	8 0	18 3		38			
4 N(CH₃)₂ †	8794	14 8	22 1						87	12	16
	879S	10 3	10 7								
4 NH₂	6819	21	37 5						56	20	34
	9077	20	31								
3 NH₂-4 C₂H₄OH	6659	48	78						44	39	65
	6761	25 6	53 5	68 5	83	87		101			
3 NHCONH (4)	7162	22 5	56 6						37	29	59
	9204	36	61								
4 NHCOCH₃-	9181	16	62						21	12	62
	9332	7 0	63								
4 CH₃NH₂	8150	34	42						13	39	48
	8184	43	53								
3 NH₂-4 OH-	5971		69						8	58	74
	6154	61 5	84								
	9075	62	68	96	98						
Tryparsamide	6466	55 4	60						0 1	68	81
	6643	78 5	85								
	6807	64	90 0								
	6821	73	89 5								

* On intraperitoneal injection into white mice

† This and following six compounds are substituted phenyl arsenoxides, only the substituent groups being indicated in the table

after intravenous injection Relatively non toxic compounds were found predominantly in the plasma, and left the blood stream at a correspondingly faster rate

The sequence of events in the blood stream was a model for what occurred in the tissues After leaving the blood, toxic compounds were firmly bound

by the organs, and relatively high levels were found in the liver and kidney 48 hours after injection; while after the injection of equimolar amounts of relatively non-toxic compounds, correspondingly small amounts were found in the tissues.

In following the excretion of arsenic the reverse picture was obtained. Toxic compounds, adhering to the tissues, were excreted only slowly, as little as 3.5 per cent appearing in the urine and stool after 48 hours; while non-toxic compounds, with relatively small affinity for the tissues, were rapidly excreted in the urine and stool, as much as 40 per cent within one hour.

The foregoing observations apply to those phenyl arsenoxides which are toxic as such, and which are bound as such by red blood cells in vitro, and presumably by tissues in vivo. They apply also to the two inorganic compounds studied, sodium arsenite and arsenate. The other types of compound studied, the arsonic acids, arsphenamines, and acid-substituted phenyl arsenoxides, are special cases, inasmuch as they are converted to other more toxic compounds in vivo.

In the case of the arsphenamines, there is good reason to believe that they owe their therapeutic activity as well as systemic toxicity to their partial oxidation in vivo to the corresponding arsenoxide (7, 8, 9). Some of these compounds are also oxidized in solutions in vitro, but not necessarily to the same degree as they are after intravenous injection. For the arsphenamines the in vitro test with red blood cells is therefore of little significance in relation to systemic toxicity.

There is considerable evidence that arsonic acids are reduced in vivo to the corresponding arsenoxides. Whether the systemic toxicity of these compounds is due solely to that conversion, or whether it rests in part on an intrinsic toxicity of the arsonic acids as such, the tissue arsenic contents 24 and 48 hours after injection were consistent with, and roughly predictable from, their LD_{50} values.

Perhaps the most interesting group of compounds studied were the acid-substituted phenyl arsenoxides. These were not bound by red blood cells in vitro to a significant degree, and would presumably not be bound by tissues in vivo. Consistent with that hypothetical lack of affinity for body cells, the five compounds in this series studied in vivo were at first rapidly excreted, at a rate exceeding that of any other phenyl arsenoxide studied. After the first few hours, however, the excretion fell off sharply, and there was a subsequent retention of arsenic, particularly in the kidney. Moreover, although these compounds proved unexpectedly toxic on systemic injection, death in white mice injected at the LD_{50} level was regularly delayed as compared with death from other arsenoxides injected at the same level. These observations suggest that most acid-substituted compounds are non-toxic as such, consistent with their demonstrated lack of affinity for red blood cells in vitro, and with their rapid initial excretion. They are, however, apparently converted to other compounds in vivo which are bound by the tissues and which are toxic. This results in a sudden decrease in the rate of excretion, and in the characteristically delayed death of mice injected at the LD_{50} level. Here then is the reverse

of the ordinary physiologic mechanism of detoxification, in that the body apparently converts a relatively innocuous compound to one which is firmly bound by the body cells, and which is toxic to the host by virtue of that combination

If arsenic compounds are toxic to the degree that they are bound, it necessarily follows that at doses which produce equivalent toxic effects, there should be equivalent amounts of arsenic in the tissues, regardless of the nature of the compound, and regardless of the absolute amounts injected This was borne out experimentally by the demonstration that three out of four arsenicals injected at the LD₅₀ level produced essentially the same arsenic concentrations in the tissues, despite the fact that these LD₅₀ doses varied five hundred fold The acid substituted 4 COOH compound gave substantially lower tissue levels, for reasons already discussed

The present data are thus consistent with the general thesis of Ehrlich that the pharmacologic effects of arsenicals are determined by their combination with specific ' arsenoceptor ' groups, whether in the tissues of the host (organotropism'), or in the invading organism ('parasitotropism") It has here been shown that the wide differences in the toxicity of a series of phenylarsin oxides are determined by corresponding differences in the amount of each bound by these ' receptors ' The wide differences in their therapeutic activity may well rest on a similarly varying combination with the receptors of the organism

There has been considerable speculation as to the nature of the arsenic recep tors in tissues and in organisms The known affinity of arsenic compounds for—SH groups suggests that similar thiol groups may be the arsenic receptors in protoplasm The extensive studies of Voegtlin and his associates (2, 3, 7, 8, 12) demonstrating the fact that thiol compounds as a group modify the biological activity of arsenicals in vitro and in vivo have led them to suggest that arsenic is a specific poison for glutathione and possibly other—SH com pounds which may occur in protoplasm, and which are essential for the metab olism of the cell A number of other workers have also demonstrated the detoxifying effect of —SH compounds on arsenic compounds, both in vitro and in vivo (13) The recent demonstration by Barron and Singer (15) that a wide variety of enzyme proteins containing —SH groups are reversibly inacti vated by arsenicals, with the simultaneous disappearance of the titratable —SH groups, strongly supports the basic thesis that —SH groups in protoplasm, whether free glutathione, fixed ' —SH groups in the sense of Hopkins and Dixon (cf Rosenthal (3)) or more specifically, free —SH groups in various enzyme proteins essential to the life of the cell, are the arsenic receptors postulated by Ehrlich The quantitatively varying effects of the same arsenic compound in different cells and of different compounds in the same cell may be a function of selective cell permeability, or may be referable to differences in affinity be tween individual arsenicals and individual —SH compounds The determination of the hydrolysis constants of a series of thioarsenites would be of interest in this connection

SUMMARY

1. In a series of phenyl arsenoxides varying twenty-fold in toxicity, the amount of each (acid-substituted compounds excepted) bound by red blood cells in vitro was in proportion to its systemic toxicity.

2. A similar variation was found in the amount of arsenical bound by the circulating red blood cells immediately after intravenous injection. The nontoxic compounds were not bound to the same degree as toxic compounds, and left the blood stream at a faster rate.

3. The amount of arsenic remaining in the liver and kidney 24 or 48 hours after the intravenous injection of arsenoxides or arsonic acids was proportional to their toxicity.

4. The rate of excretion of phenyl arsenoxides (acid-substituted compounds excepted) was also a function of their toxicity. The non-toxic compounds, not bound by body cells, were excreted rapidly; while the toxic compounds were excreted slowly, in inverse proportion to their toxicity.

5. At dosages which produced equivalent toxic effects (the LD_{50} level), tryparsamide, phenyl arsonic acid and phenyl arsenoxide resulted in comparable tissue levels, despite a 500-fold difference in absolute arsenic dosage.

6. It is therefore suggested that the varying systemic toxicity of arsenicals is primarily determined by the varying degree to which they are bound by, and thus block, essential functional groups in vital organs. The chemical nature of these groups is discussed in the text.

7. Acid-substituted phenyl arsenoxides are only an apparent exception to this generalization. Although fairly toxic, they were bound to only a minimal degree by red blood cells in vitro or in vivo. After intravenous injection, they were at first excreted rapidly, as much as 40 per cent appearing in the urine in one hour. The excretion was, however, abruptly curtailed after approximately four hours; and death in white mice injected at the LD_{50} level was characteristically delayed as compared with death resulting from other phenyl arsenoxides. It seems probable that most acid-substituted phenyl arsenoxides are not toxic as such, consistent with their lack of affinity for red blood cells, and their initially rapid excretion. The sudden curtailment of urinary excretion, and the delayed death of mice, suggest that they are converted by the body to other compounds which can combine with vital chemical groupings in the tissues, and which are toxic by virtue of that combination.

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THE DETERMINATION OF SALICYLIC ACID IN PLASMA[1]

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The use of salicylates in rheumatic fever has been empirical, with no concensus of opinion as to the dosage required or as to its value in modifying the progress of the disease process. Recent work, however, has indicated that high concentrations of salicylate maintained in the plasma for a long period of time do markedly modify the sterile inflammatory process and inhibit the development of cardiac disease (1).

Knowledge of the plasma concentration of salicylate is essential for the construction of rational regimes of therapy. The methods for estimating salicylate that have been previously described are not precise (2). A simple procedure for routine use is described here. This involves a separation of the salicylic acid from plasma by extraction into ethylene dichloride and its return to an aqueous phase as the colored iron complex which is determined colorimetrically.

REAGENTS. 1. Standard Salicylic Acid Solution, 100 mgm. per 100 ml. One hundred and sixteen mgm. of sodium salicylate are dissolved in exactly 100 ml. of water. This standard is quite stable when stored in the refrigerator. Working standards are prepared by dilution with water.

2. $6 N$ HCl.

3. Ethylene dichloride.

4. 1% $Fe(NO_3)_3$ in 0.07 N HNO_3. This concentration of nitric acid has been selected since it is sufficiently strong to prevent hydrolysis of the iron salt, and yet not impair the iron-salicylic acid reaction.

PROCEDURE. Add 2 ml. of plasma, or 1 ml. of plasma and 1 ml. of water, to 0.5 ml. of $6 N$ HCl and 30 ml. of ethylene dichloride in a 60 ml. glass-stoppered pyrex bottle. Shake vigorously for 5 minutes, preferably on a shaking apparatus. Transfer the mixture to a 35 ml. thick-walled test tube and centrifuge for 5 minutes at moderate speed. Remove the supernatant aqueous layer by aspiration. Transfer exactly 20 ml. of the ethylene dichloride layer to a dry 60 ml. glass-stoppered bottle and add 10 ml. of water and 0.25 ml. of the iron reagent. Shake for 5 minutes. Transfer at least 6 ml. of the supernatant aqueous layer to a colorimeter tube. Read in the colorimeter, using a filter with maximal transmission at 540 mu.

Standard Curve. The distribution coefficient of salicylic acid in an ethylene dichloride acidified water system is such that at room temperature, with volumes of 30 and 2.5 ml. respectively, 90% of the salicylic acid is in the organic phase. This coefficient is highly reproducible and does not appear to be sensitive to small changes in temperature. Stan-

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dards are prepared by taking 1 ml of standard salicylic acid solution 1 ml of water, adding 0 5 ml of 6 N HCl and handling in the same manner as the plasma determination A standard curve is constructed on semi logarithmic paper, with per cent transmission plotted against milligrams per cent of salicylic acid

RESULTS Estimations of salicylic acid added in various amounts to human plasma were performed to test the accuracy of the method The data in Table I indicate that salicylic acid added to plasma is recoverable with satisfactory

TABLE 1
Recovery of salicylic acid added to plasma

SALICYLIC ACID ADDED	SALICYLIC ACID FOUND	RECOVERY
mgm	*mgm*	*per cent*
0 050	0 050	100
	0 047	94
	0 052	104
	0 054	108
	0 050	100
	0 050	100
0 100	0 095	95
	0 100	100
	0 100	100
	0 103	103
	0 098	98
	0 098	98
0 200	0 203	102
	0 198	99
	0 198	99
0 300	0 295	98
	0 292	97
	0 300	100
	0 303	101
	0 303	101
0 500	0 510	102
	0 517	103
	0 480	96
	0 480	96

precision Analyses run on single samples of plasma over a period of several days always gave highly reproducible results and it may be concluded that salicylic acid in plasma is stable when stored in the refrigerator

Specificity The absence of detectable blanks in salicylate free plasma indicates that the constituents of normal plasma do not participate to a measurable extent in these reactions This simplifies an examination of the specificity of the procedure The degree of specificity is dependent only upon the extent to which the metabolic products of salicylic acid itself are excluded in

the final colorimetric estimation. Two such metabolic products are present in large amounts in the urine of human subjects receiving sodium salicylate (3). Of these, only salicyluric acid is soluble in ethylene dichloride and reacts with the iron. Its presence in appreciable amounts in the plasma would therefore be a source of error in the estimation of salicylic acid.

The difference in the distribution coefficients of salicylic acid and salicyluric acid in the ethylene dichloride-water and carbon tetrachloride-water systems was used to assay the possible error in plasma determinations due to the presence of salicyluric acid. One hundred micrograms of salicyluric acid added to 1 ml. of plasma were found to be equivalent to 35 micrograms of salicylic acid in the routine procedure. However, when carbon tetrachloride was substituted for the ethylene dichloride the same amount of salicyluric acid was equivalent to only

TABLE 2

Comparison of salicylic acid determinations using ethylene dichloride and carbon tetrachloride as organic solvents

These plasma specimens were obtained from patients receiving therapeutic doses of sodium salicylate.

SPECIMEN	ETHYLENE DICHLORIDE	CARBON TETRACHLORIDE
	mgm. %	*mgm. %*
1	32.7	31.0
2	22.5	22.3
3	32.6	32.1
4	24.5	24.0
5	34.7	33.4
6	43.8	41.9
7	36.6	34.2
8	30.8	29.3
9	27.0	26.4
10	38.3	36.9
11	25.8	26.5
12	24.0	25.3
13	21.4	21.6
14	19.9	19.7

5 micrograms of salicylic acid. On the other hand the distribution ratio of salicylic acid between acidified water and carbon tetrachloride is the same order as that which obtains with ethylene dichloride. These circumstances favor the use of carbon tetrachloride in appraising the specificity of the routine procedure.

Salicylic acid determinations were made on a series of plasma samples from patients on sodium salicylate therapy. The estimations were made using both ethylene dichloride and carbon tetrachloride as the organic solvents. The results of this estimation are summarized in Table II. The differences between the salicylic acid concentrations determined with ethylene dichloride and with carbon tetrachloride were invariably small, the maximum being 7 per cent. It may be concluded from this finding that the error due to the presence of salicyluric acid is negligible in the case of plasma. Other known derivatives of salicylic acid do

not constitute a source of error in the procedure in that they are either not extractable from plasma by ethylene dichloride or do not form stable colored complexes with iron [3]

The presence of large quantities of salicyluric acid in the urine precludes the use of the simple procedure for the estimation of salicylic acid in this biological fluid

SUMMARY

A method is described for the estimation of salicylic acid in plasma   The salicylic acid is separated from plasma by extraction with ethylene dichloride from an acid medium   It is then returned as the colored iron complex to an aqueous phase and determined colorimetrically. The procedure is simple and permits the routine estimation of the plasma concentrations of salicylic acid achieved with the usual therapeutic doses

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[3] It is not advisable to substitute carbon tetrachloride for ethylene dichloride in the routine procedures since the distribution ratio of salicylic acid in the carbon tetrachloride and water system is different than in a carbon tetrachloride plasma system and both are somewhat variable   The observations using carbon tetrachloride required the simultaneous construction of standard curves using salicylic acid in plasma for the standard solutions

A PHARMACOLOGIC STUDY OF 5 ALLYL-5 Δ²-CYCLOPENTENYL BARBITURIC ACID (CYCLOPAL)

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Sixty five new barbituric acid derivatives prepared by Blicke and Zienty (1) and Centolella, Nelson and Kolloff (2) have been evaluated in this laboratory by determining their $\frac{L D 50}{A D 50}$ ratios and types of response in rats. Of the different series of barbiturates studied those containing the cyclopentenyl radical showed the most promise. Cyclopal (5 allyl-5 Δ² cyclopentenyl barbituric acid), selected as the best of the cyclopentenyl series, has been studied more extensively in rats, rabbits and dogs by intraperitoneal, subcutaneous and oral administration in parallel with the U S P barbiturates and several accepted in N N R. Because Cyclopal appears to be most like Pentobarbital U S P, the actions of the two are compared in the experiments herein reported which are limited to acute and chronic studies on rats and dogs following oral administration.

ACUTE TOXICITY. *Methods employed.* Male rats between 150 and 225 grams in weight and mature healthy dogs of both sexes which had not had access to food for a period of approximately twenty hours prior to treatment were used. The barbiturates were administered by stomach tube. Rats were given solutions of the sodium salts of Cyclopal and Pentobarbital U S P containing the equivalent of 25 mgm of the free acid per cc and dogs were given similar solutions containing 50 mgm of the free acid per cc. Duration of anesthesia' was taken as the time following administration of the barbiturates during which pinching the tail failed to cause the rats to right themselves and pressure on the thigh muscles failed to arouse the dogs.

Experimental results. Rats and dogs. The results obtained are shown as follows

BARBITURIC ACID	NUMBER OF ANIMALS	A D 50	L D 50	L D 50 / A D 50	INDUCTION TIME		DURATION	
					A D 50	50% L D 50	A D 50	50% L D 50
		mgm/kgm	mgm/kgm		min	m n	hrs	hrs
Rats								
Cyclopal	113	75	205	2 7	20		1 3	
Pentobarbital	36	50	118	2 4	25		1 1	
Dogs								
Cyclopal	38	27	105	3 9	23	32	1 5	14 0
Pentobarbital	28	18	65	3 6	24	35	1 2	5 5

They indicate that in both species Cyclopal and Pentobarbital possess approximately the same $\frac{\text{L.D.50}}{\text{A.D.50}}$ ratios. Between 50 and 60 per cent more Cyclopal than Pentobarbital is required to obtain the A.D.50 and L.D.50 in both rats and dogs. Onset of "anesthesia" is about the same with both barbiturates but duration is longer following Cyclopal.

CHRONIC TOXICITY. *Methods employed.* Fifty 150-gram male rats and ten mature, healthy dogs (male and female) were used for the comparative study of the chronic toxicity of Cyclopal and Pentobarbital U.S.P. Twenty rats and four dogs were used for the study of each drug. Ten of the rats and two of the dogs served as controls. The barbituric acids were administered after a twenty hour fast as solutions of their sodium salts (25 mgm. and 50 mgm. of the barbituric acid per cc. for rats and dogs respectively) by stomach tube on alternate days three times weekly for from twenty-three to twenty-eight weeks. The doses for rats were adjusted at each administration to an amount anticipated to be the 50 per cent "anesthetic" dose for the group. For dogs, on the other hand, the dose for each was adjusted at each administration to an amount thought to be just large enough to cause "anesthesia". The body weight and quality and length of induction and duration of "anesthesia" were recorded for each animal. In Fig. 1 the data for the experiment with rats are shown by plotting (for the thirteen survivors of each group) averages of body weight, dose of barbiturate, per cent "anesthetized", induction time and duration of "anesthesia" on the ordinate against time in weeks on the abscissa. In Fig. 2 similar data are shown for the experiment with dogs.

Experimental results (Rats). Thirteen rats of each of the treated groups and nine in the control group survived the experiment. The Pentobarbital fatalities occurred between the first and ninth weeks while those in the Cyclopal group were evenly distributed between the second and twenty-sixth weeks. Some deaths can be attributed in part to respiratory infection while others may have been due to the toxic action of the barbiturates, although grossly no tissue damage was apparent. The organs of those rats surviving treatment likewise appeared in no way to differ grossly from those of the controls.

From Fig. 1 it can be seen that "anesthesia" was fairly well maintained on an average of approximately 50 per cent for both groups and that neither suffered from the effects of continued barbiturate administration as far as growth was concerned.

The arithmetical means of the chronic A.D.50 of Cyclopal and Pentobarbital are 91 ± 8.2 and 49 ± 5.6 mgm. per kgm. respectively, indicating about an equal spread on a percentage basis. There was a tendency, however, for the rats to become slightly more susceptible to the action of Pentobarbital and more refractory to the action of Cyclopal.

The average induction times were twenty-two and eleven minutes for Cyclopal and Pentobarbital respectively. Onset of "anesthesia" was remarkably uniform over the entire period of the experiment. The average duration of "anesthesia", on the other hand, while approximately 60 per cent longer following Cyclopal (112 minutes) than following Pentobarbital (69 minutes) showed considerable variation for both drugs over the six month period. In general, duration of "anesthesia" appears to parallel dosage.
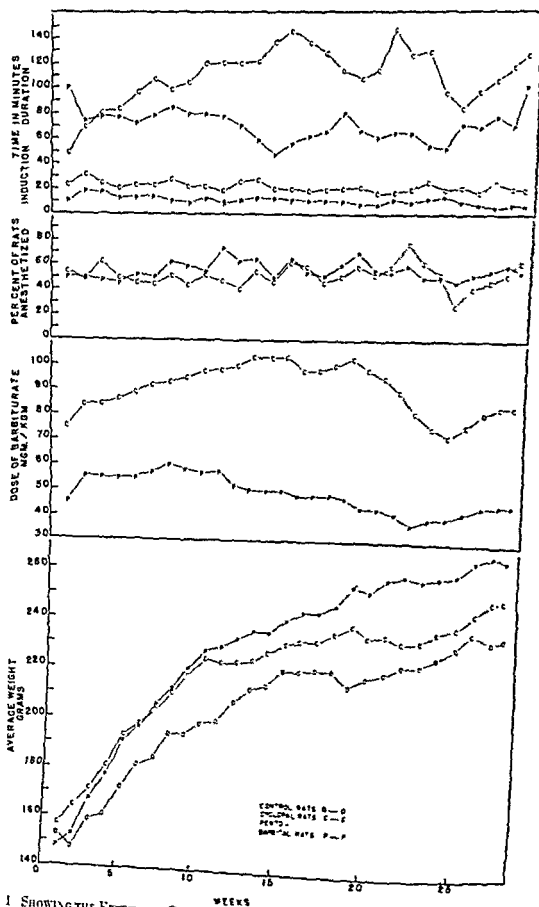
Fig 1 Showing the Effects on Groups of Male Rats of the A D 50 of Pentobarbital and Cyclopal Administered Orally Three Times Weekly for Twenty-eight Weeks (See Text for Details)
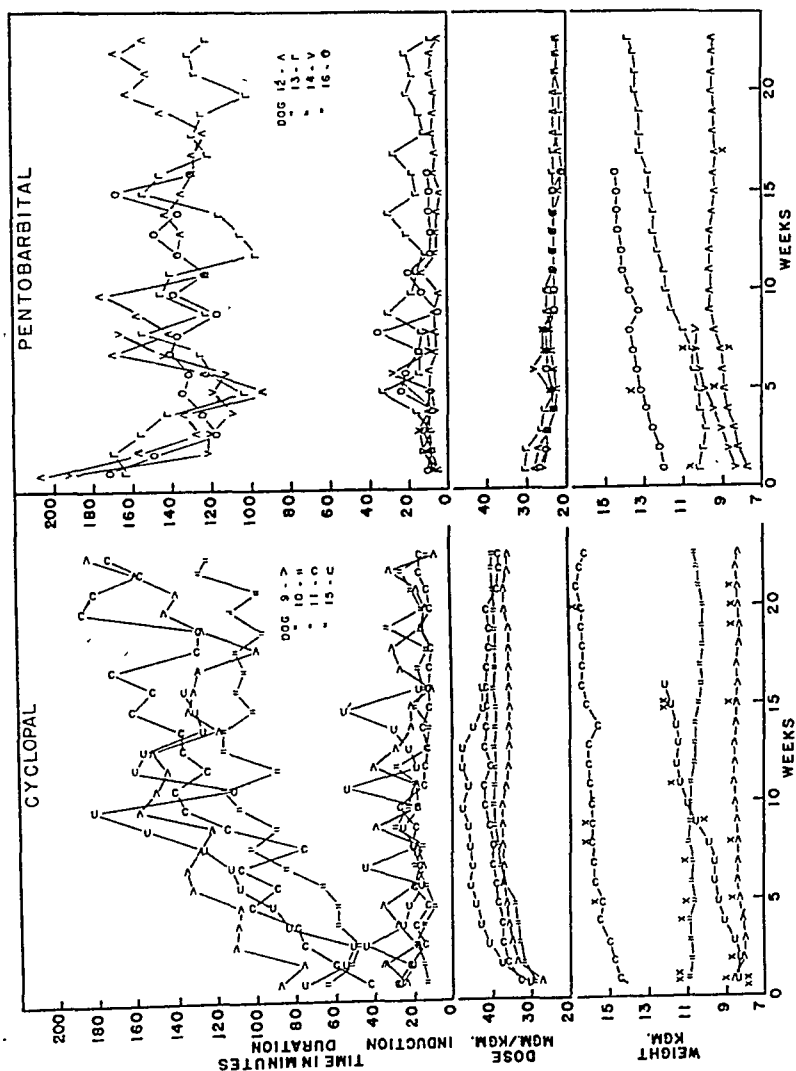
FIG. 2. SHOWING THE EFFECTS ON INDIVIDUAL DOGS OF THE A.D.100 OF PENTOBARBITAL AND CYCLOPAL ADMIN-ISTERED ORALLY THREE TIMES WEEKLY FOR TWENTY-THREE WEEKS (SEE TEXT FOR DETAILS)

(Dogs) The general good health of the dogs at the termination of the experiment is reflected by the body weight curves plotted in Fig 2   Dog 14 (Pentobarbital) was the only fatality   His death in the eighth week, two days following his last administration of Pentobarbital, is attributed to respiratory infection   Dogs 15 (Cyclopal), 16 (Pentobarbital) and 37 (control) were sacrificed after sixteen weeks of treatment and the remaining six dogs after twenty three weeks of treatment   Doctors Walter Schiller of Cook County Hospital and M B Jacobs of Loyola University, who kindly made the gross and microscopic examinations on these dogs, concluded that the tissues[1] of the treated dogs differed only within normal limits from those of the controls and that there was no evidence of degenerative or other pathologic changes   Corroborating evidence that the liver, which is probably the site of detoxification of Cyclopal and Pentobarbital, was undamaged by the barbiturates was obtained by use of the bromsulphalein liver function test during the last two weeks of treatment   The rate of disappearance of the dye from the blood stream of the test animals in no way differed from its rate of disappearance from the blood stream of the control animals

The dosage curves in Fig 2 show the weekly averages of the minimum dose required to "anesthetize" each dog   It is to be expected that there would be individual differences in susceptibility to both drugs   However, with the exception of dog 15, who was particularly resistant, the minimum doses required to produce anesthesia' throughout the experiment are in good agreement

The Cyclopal treated dogs uniformly became refractory to continued treatment   On an average, however, maximum tolerance (approximately 43 per cent more Cyclopal than was required at first) was reached in about six weeks and thereafter there was little change in effective dosage   No refractoriness, on the other hand, was exhibited by the group of dogs on Pentobarbital   Maintenance of approximately 100 per cent response which was of fairly uniform duration was possible with the Pentobarbital dogs in spite of a gradual though not marked decrease in dose

The average time of induction of "anesthesia" of dogs receiving both barbiturates varied considerably from week to week as shown in Fig 2   There was, however, no trend toward lengthening or shortening of induction time observed on any dog   The arithmetical mean of all induction times for Cyclopal was twenty one minutes and for Pentobarbital thirteen minutes

The duration of anesthesia' of the Cyclopal treated dogs increased over the entire period of the experiment   The rate of increase was greatest, however, for the first eight weeks during which time it was found necessary to gradually increase the dosage in order to induce anesthesia"   The average length of 'anesthesia" for the Pentobarbital dogs after an initial high value fell to a level which was fairly well maintained

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[1] The following tissues were examined microscopically esophagus stomach, duodenum jejunum colon pancreas liver gall bladder kidney urinary bladder spleen, pituitary thyroid adrenal striated muscle artery myocardium lung peripheral nerve brain (cerebellum and cortex) bone marrow ovary and testis

In order to indicate the frequency at which "anesthesia" occurred for each dog throughout the experiment, beside each point on the weight curves, which represent the average values of three alternate days, are placed "x" and "xx" if no "anesthesia" occurred after one and two of the three administrations respectively. "Anesthesia" resulted following each of the three administrations on all points unmarked.

The quality of response elicited by these barbiturates appears to be equal when judged by the smoothness of induction of "anesthesia", the depth of "anesthesia" and the rate of disappearance of hypnotic symptoms. Slight excitement was frequently observed during onset of "anesthesia". In general, however, induction was remarkably smooth. Depth of "anesthesia" was fairly uniform over the entire period of the experiment. Recovery from the hypnotic effects of both drugs was very rapid in marked contrast to the rate of recovery from barbiturates predominantly eliminated by excretion in the urine.

EXCRETION STUDIES. *Methods employed.* The urine of dogs receiving Cyclopal, Pentobarbital and Barbital was examined in order to ascertain what part of the ingested or injected material was eliminated from the body by the kidneys. The methods of isolation and purification studied were those of Koppanyi et al. (3), Shonle et al. (4) and Brundage and Gruber (5). The colorimetric estimation of the barbiturate extracted from the urine as used in the methods employed by Koppanyi et al. and Brundage and Gruber proved unsatisfactory in our hands partly because of the interference of urine pigments and partly because, as Kozelka, Nelson and Tatum (6) have reported, the color intensity was not proportional to the barbiturate concentration. Best results were obtained with urine samples when the product isolated by the method of Brundage and Gruber was sublimed and its hypnotic activity compared with the hypnotic activity of the pure barbiturate when both were injected into 50 gram rats.

Experimental results. Eleven experiments on eight dogs to which the barbiturates were administered in doses ranging from subhypnotic to the L.D.50 indicated that at most only 2.5 per cent of the administered Cyclopal or Pentobarbital was present in the urine excreted in the first fifty hours. On the other hand, 25 per cent of a subhypnotic intraperitoneal dose of Barbital was recovered from urine collected for seventy-two hours. These results are in agreement with elimination studies reported for Cyclopal by Dille and Kipple (7) using rabbits and indicate that Cyclopal and Pentobarbital differ from Barbital in not being eliminated to an appreciable extent in the urine.

CONCLUSIONS

In rats and dogs Cyclopal has a very good "margin of safety". It is approximately equal to that of Pentobarbital U.S.P. Cyclopal's minimal effective dose is approximately 50 per cent greater than that of Pentobarbital and its effectiveness lasts for a somewhat longer period of time. The interval between the end of "anesthesia" and the time of complete freedom from hypnotic symptoms is short for both drugs. Cyclopal and Pentobarbital are not excreted as such in amounts greater than 2.5 per cent of the quantity administered in the urine of dogs which probably indicates that these barbiturates are broken down in the body.

In the chronic experiments Cyclopal behaved like Pentobarbital in that it produced no detectable tissue damage and at the conclusion of the experiment had caused no change in the deportment of the animals from the normal   Cyclopal differed from Pentobarbital in these respects   The animals became somewhat more refractory to Cyclopal and the period of 'anesthesia" increased with the necessarily larger doses required to produce it   On the other hand, they became slightly susceptible to the action of Pentobarbital but the duration of "anesthesia" remained substantially the same

SUMMARY

Cyclopal (5 allyl 5 Δ° cyclopentenyl barbituric acid) selected as the best of a series of cyclopentenyl barbiturates, has been compared with Pentobarbital U S P in rats and dogs from the standpoint of acute and chronic oral toxicity, effectiveness and excretion

The $\dfrac{L\ D\ 50}{A\ D\ 50}$ ratios for Cyclopal and Pentobarbital respectively were found to be $\dfrac{205}{75}$ and $\dfrac{118}{50}$ for rats and $\dfrac{105}{27}$ and $\dfrac{65}{18}$ for dogs When dogs were given 50 per cent of the L D 50 the onset of anesthesia was approximately one half hour for both drugs   The duration of anesthesia at this dosage level was longer for Cyclopal (fourteen hours) than for Pentobarbital (five and one half hours)

Using the minimum dose response relationship as a criterion both rats and dogs in the chronic study became somewhat refractory to Cyclopal and slightly susceptible to Pentobarbital   The degree of response i e , duration of 'anesthesia' appeared in general to be related directly to the dose

The quality of response was the same for both Cyclopal and Pentobarbital as judged by smoothness of onset of   anesthesia", depth of   anesthesia' and rapidity of recovery from hypnotic symptoms

In dogs absence of degenerative or pathological changes normal liver function general good health and normal deportment were observed at the end of five months of treatment with Cyclopal and Pentobarbital (A D 100 on alternate days)

Hypnotic material excreted in the urine of dogs receiving doses as large as the L D 50 does not amount to more than 2 5 per cent of the ingested Cyclopal or Pentobarbital

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THE INFLUENCE OF TEMPERATURE ON THE ACTION OF DIGITOXIN AND POTASSIUM ON STRIATED MUSCLE

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Certain of the effects of the digitalis glycosides on the function of striated muscle resemble those resulting from an excess of potassium. As a result of studies on the action of ouabain on the energetics of contraction of the isolated frog's sartorius muscle, Cattell (1) concluded that the characteristic changes "are due to the formation or liberation of some substance which readily diffuses from the muscle" and "that these changes are consistent with those caused by the escape of potassium from the cell."

The phenomenon with which we are here concerned is the reversible loss of excitability in the frog's sartorius muscle resulting from an excess of potassium, originally discovered by Sereni (2) and studied in detail by Dulière and Horton (3) and by Solandt (4). A similar effect due to an action of the digitalis glycosides has been reported by Cattell (1). It is the purpose of the present communication to present the results of studies on the influence of temperature on the reversible loss of excitability in striated muscle treated with potassium or digitoxin and to consider further the mechanism of digitalis action.

METHODS. *A double sartorius preparation from the frog was employed in all of the* experiments. It was immersed in Ringer's solution (NaCl 0.675 per cent, CaCl$_2$ 0.020 per cent, KCl 0.015 per cent, phosphates 0.10 per cent to give a pH value of 7.2) for at least one hour to assure a uniform response of the preparation and equalization of the temperature. The preparations were set up in a moist chamber, which was immersed in a large vessel filled with water at the desired temperature. Oxygen was bubbled slowly and continuously through the chamber. The contractions were recorded on a kymograph through an isometric lever.

In the experiments devised to determine the effect of digitoxin at various temperatures, the Ringer's solution was removed from the chamber and replaced by a similar medium containing digitoxin (1 to 2 parts in one million), in which the preparation was for a variable time, generally twenty minutes. The length of time that the preparation was exposed to digitoxin did not influence the result provided it was upwards of ten minutes. After withdrawl of this solution, with the muscle in an atmosphere of oxygen, a twitch was recorded every five minutes until a marked depression of excitability occurred, indicated by a tension drop to well below 50 per cent of the initial value. Ringer's solution was returned to the chamber to permit a return of normal excitability. This procedure, alternating between digitoxin and unmodified Ringer's, was repeated several times on the same preparation; in some instances as many as eight exposures to digitoxin were performed.

In the series of experiments devised to determine the effect of temperature on the action of potassium, the Ringer's solution was removed from the chamber and replaced by one to which KCl (5 to 10 times the amount present in the balanced Ringer's) had been added. This was allowed to act for from 10 to 30 minutes and, after withdrawl of the fluid, the

twitch tensions were obtained at regular intervals while the muscles remained in an atmosphere of moist oxygen   As the muscles were not washed, the potassium which had penetrated the interior or had remained on the surface of the muscles was presumably present throughout the course of the experiment   The effect of repeated exposure to potassium excess at different temperatures was studied   The results were compared with those obtained on the preparations which were treated with digitoxin

RESULTS   *Digitoxin*   The data from six experiments showing the effect of repeated short exposures to digitoxin (at room temperature) on the tension of the contracting sartorius muscle are presented in table 1   From these data it is clear that the time required to reach 50 per cent tension is not a constant value in the same preparation kept at room temperature   The time for half tension following the second short exposure was always less than that required for the same effect following the first immersion   The time following the third exposure was slightly less than the second, and the fourth about equal to the third   Subsequent exposures varied but slightly in time for the development of half

TABLE 1

Repeated exposure to digitoxin at constant temperature

EXPERIMENT	TIME IN MINUTES FOR 50 PER CENT REDUCTION IN TENSION FOLLOWING REPEATED EXPOSURE TO 1 1 000 000 DIGITOXIN			
	1st	2nd	3rd	4th
F 12	39	31	39	
F 14	76	56	48	35
F 15	50	46	51	
F 17	108	70	80	
F 23	138	60	42	36
F 27	90	55	38	47
Average	83 5	53	49 7	
Per cent	100	63 5	59 5	

tension   The early twitches of the series usually showed an increase in tension which followed a rather definite pattern   The increased tension was generally greatest following the first exposure and the time for this development was longer than that which followed the second and subsequent exposures   This augmentation gradually decreased, and, in many instances, did not appear following the fourth and subsequent exposures   These changes are similar to those reported by Guttman and Cattell (5)

The data from six experiments showing the effect of temperature on tension following repeated short exposures to digitoxin are presented in table 2   The time for the development of half tension is much increased as a result of lower temperature (the lower temperature was 10 degrees C less than the higher temperature, which ranged between 20–27 degrees C)   These data indicate a Q_{10} of nearly 2

POTASSIUM   The effect of repeated exposure of the frog sartorius preparation to K excess in Ringer's solution was reported by Guttman and Cattell (5)   It

was observed that "when the concentration of K is raised to a value higher than about five times that normally present in Ringer's solution, i.e., increased from 8 mgm./100 cc. to almost 40 mgm./100 cc., a series of characteristic changes occur which closely resemble those observed following immersion in dilute ouabain or digitoxin solutions." The data presented in table 3 indicate the time

TABLE 2

Repeated exposure to digitoxin at different temperatures

| EXPERIMENT | TIME IN MINUTES FOR 50 PER CENT REDUCTION IN TENSION FOLLOWING REPEATED EXPOSURE TO 1:1,000,000 DIGITOXIN | | |
	1st	2nd	3rd
F 21	121 (27)*	140 (17)*	80 (27)*
F 52	65 (27)	83 (17)	43 (27)
F 54	80 (26)	120 (16)	70 (26)
F 56	125 (20)	110 (10)	65 (20)
F 57†	63 (20)	53 (10)	25 (20)
F 58	83 (24)	150 (14)	23 (24)
Average	89.5 (24)	109.3 (14)	51 (24)
Per cent	100	122	57

* Temperature in degrees C.
† 1:500,000 digitoxin.

TABLE 3

Repeated exposure to potassium at constant temperature

| EXPERIMENT | POTASSIUM | TIME IN MINUTES FOR 50 PER CENT REDUCTION IN TENSION FOLLOWING REPEATED EXPOSURE TO POTASSIUM | | | |
		1st	2nd	3rd	4th
	mgm. per 100 cc.				
B 3	47.1 (6x)*	28	17		
B 5	39.3 (5x)	53	40		
B 6	39.3 (5x)	72	40		
F 44	62.9 (8x)	22	10	13	16
F 45	62.9 (8x)	19	17	14	12
F 49	62.9 (8x)	30	10	18	6
Average.		37	22		
Per cent.		100	59.3		

* Potassium concentration in multiples of that present in normal Ringer's solution.

required to elicit a 50 per cent reduction in muscle response (twitch tension) when the soluble sartorius preparation is repeatedly exposed to an excess of potassium at a constant temperature (about 22–24 degrees C.). The average (6 experiments) time for this reduction, following the second exposure, was about 60 per cent of the control value. Table 4 consists of data indicating the effect of temperature on the response of the sartorius to Ringer's solution preparation

with an excess of potassium   In the seven experiments, the second exposure was at a temperature ten degrees C lower than the first, and the average time to half twitch tension was about 70 per cent of that at the higher temperature These data demonstrate that Q_{10} values for preparations exposed to potassium excess Ringer's solutions are very small, about 1 2

Discussion   The experiments described above demonstrate a positive temperature coefficient for the action of digitoxin resulting in loss of excitability in isolated striated muscle   This is in accord with data presented by MacKenzie (6), Cohn and Jameson (7), Stone, Phillips and Bliss (8), McGuigan (9), Fischer (10), Sollmann, Mendenhall and Stingel (11), Baker (12), Haskell (13), Gunn (14), Hirschfelder, Bicek, Kucera and Hanson (15) and Jameson (16), who administered various digitalis glycosides to intact animal preparations including

TABLE 4

Repeated exposure to potassium at different temperatures

EXPERIMENT	POTASSIUM	TIME IN MINUTES FOR 50 PER CENT REDUCTION IN TENSION FOLLOWING REPEATED EXPOSURE TO POTASSIUM					
		1st		2nd		3rd	
	mgm per 100 cc						
F 40	30 3 (5x)*	65	(27 5)	55	(17 5)	40	(27 5)
F 41	30 3 (5x)	102	(29 5)	40	(19 5)	25	(29 5)
F 43 A	62 9 (8x)	22	(27 5)	20	(17 5)	18	(27 5)
F 46	62 9 (8x)	13	(27)	14	(17)	13	(27)
F 47	62 9 (8x)	16	(27)	14	(17)	12 5	(27)
F 48	62 9 (8x)	16 8	(27)	14	(17)	12 5	(27)
F 43 B	62 9 (8x)	48	(27 5)	38 5	(17)	27 5	(27 5)
Average		40 4	(27 4)	29 9	(17 4)	21 2	(27 4)
Per cent		100		70 4		52 5	

* Potassium concentration in multiples of that present in normal Ringer s solution

the frog, cat, dog and rabbit and observed the effect of temperature on the digitalis action on the heart

These studies (6–16) all indicated a positive temperature coefficient for the action of digitalis on the rhythmically beating heart and support Windaus (17) who postulated a chemical action between cardiac glycosides and muscle   The work of Cattell (1) indicates that inorganic ions are also concerned in the mechanism of digitalis action   Ouabain is effective in producing a reversible inexcitability in skeletal muscle presumably by enabling potassium to leave the myofibrillae and enter the intercellular spaces, similar to the action of potassium reported by Dulière and Horton (3) and has been further supported by potassium determinations of Ouabain treated muscles by Cattell and Goodell (19)

Potassium excess in Ringer's solution mimics the inexcitability curve of the digitalis glycosides and it was on this score that the present observations on the effect of temperature are of interest   It is now demonstrated that the effect of potassium excess is not altered by temperature to an extent comparable to the

was observed that "when the concentration of K is raised to a value higher than about five times that normally present in Ringer's solution, i.e., increased from 8 mgm./100 cc. to almost 40 mgm./100 cc., a series of characteristic changes occur which closely resemble those observed following immersion in dilute ouabain or digitoxin solutions." The data presented in table 3 indicate the time

TABLE 2

Repeated exposure to digitoxin at different temperatures

EXPERIMENT	TIME IN MINUTES FOR 50 PER CENT REDUCTION IN TENSION FOLLOWING REPEATED EXPOSURE TO 1:1,000,000 DIGITOXIN					
	1st		2nd		3rd	
F 21	121	(27)*	140	(17)*	80	(27)*
F 52	65	(27)	83	(17)	43	(27)
F 54	80	(26)	120	(16)	70	(26)
F 56	125	(20)	110	(10)	65	(20)
F 57†	63	(20)	53	(10)	25	(20)
F 58	83	(24)	150	(14)	23	(24)
Average	89.5	(24)	109.3	(14)	51	(24)
Per cent . ..	100		122		57	

* Temperature in degrees C.
† 1:500,000 digitoxin.

TABLE 3

Repeated exposure to potassium at constant temperature

EXPERIMENT	POTASSIUM	TIME IN MINUTES FOR 50 PER CENT REDUCTION IN TENSION FOLLOWING REPEATED EXPOSURE TO POTASSIUM			
		1st	2nd	3rd	4th
	mgm per 100 cc				
B 3	47.1 (6x)*	28	17		
B 5	39.3 (5x)	53	40		
B 6	39.3 (5x)	72	40		
F 44	62.9 (8x)	22	10	13	16
F 45	62.9 (8x)	19	17	14	12
F 49	62.9 (8x)	30	10	18	6
Average.		37	22		
Per cent.		100	59.3		

* Potassium concentration in multiples of that present in normal Ringer's solution.

required to elicit a 50 per cent reduction in muscle response (twitch tension) when the soluble sartorius preparation is repeatedly exposed to an excess of potassium at a constant temperature (about 22–24 degrees C.). The average (6 experiments) time for this reduction, following the second exposure, was about 60 per cent of the control value. Table 4 consists of data indicating the effect of temperature on the response of the sartorius to Ringer's solution preparation

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TISSUE ALDEHYDES AND THEIR REACTION WITH AMINES

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Feulgen and Voit (1) used the fuchsin sulfurous acid (FSA)[2] staining reaction to demonstrate the presence of tissue aldehydes to which they referred as "plasmal." The plasmal was liberated from a precursor called "plasmalogen," either by the action of acids or by mercuric chloride. Platinic chloride, Verne (2), and gold chloride, Oster and Schlossmann (3), were also found capable of splitting the plasmalogen complex. Feulgen, Imhaeuser and Behrens (4) found that aldehydes obtained from horse muscle were a mixture of stearal and palmital and Feulgen and Bersin (5) showed them to be in an acetal-like combination with glycerophosphocholamine. Anchel and Waelsch (6) obtained aldehydes of the higher fatty acids from a variety of tissues. Acetal phosphatides have been synthesized by Bersin, Moldtmann, Nafziger, Marchand and Leopold (7). Feulgen and Imhaeuser (8) and Feulgen and Gruenberg (9) adapted the FSA reagent in the quantitative colorimetric determination of plasmal in lipoid mixtures and in organs.

The distribution of aldehydes in various tissues and their localization in the cells of different organs have been investigated by Imhaeuser (10), Verne (11), Becher (12) and Voss (13, 14). Stepp, Feulgen and Voit (15) described the occurrence of tissue aldehydes in protozoa, in several non-vertebrates and in the tissues of all vertebrates from fish to man. The intensity of the stain obtained by them with FSA varied in different organs and in different species. The lightest stain was found in sheep tissues. The intensity of the stain for human tissue was intermediate and the deepest stain was found in beef tissues. They concluded that herbivores must produce the aldehydes themselves since they did not find any in plants. In carnivores the plasmal content of the blood dropped from 24.6 mgm. per liter to 9.0 mgm. per liter, after a month on a meat free diet.

According to Feulgen, Imhaeuser and Westhus (16), ingested plasmalogen increased the aldehyde reaction in the serum, but ingested plasmal did not do so. Only that part of the ingested plasmal which escaped splitting by the hydrochloride acid of the stomach participated in the subsequent plasmalogenemia. The aldehyde reaction in the serum was increased up to one hour after the intravenous administration of plasmalogen. Plasmal given intravenously did not influence the aldehyde content of the serum.

It is the purpose of this report to show that an important detoxification

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[1] Upjohn Fellow, 1942–1943.

[2] Schiff's reagent is very sensitive, but it is not specific for aldehydes, as it gives positive results with certain ketones.

mechanism is the formation within the cell of a Schiff's base, by the interaction between an amine and an aldehyde $R_1CHO + R_2$ phenyl-$NH_2 \rightarrow R_1CH =$ N-phenyl-R_2. The limits within which this reaction is possible in the tissues studied have been delineated by the use of a large variety of amines. It was shown by the use of the FSA reaction that both para-aminobenzoic acid and the sulfonamides will combine with tissue aldehydes. It is suggested that this competition extends to bacterial substance as well, in which case the oxidation of the metabolically necessary para-aminobenzoic acid may be interfered with by the sulfonamides and bacteriostasis ensue. Conversely, the bacteriostatic effects of the sulfonamides would be interfered with by an excess of para amino benzoic acid.

EXPERIMENTAL Rats guinea pigs rabbits, cats and dogs were used in this study. The liver and kidney tissues were obtained from normal animals after stunning by a blow on the head or while under ether or sodium pentobarbital anesthesia and subsequent bleeding. Pieces of the tissues were put into saline and frozen sections, 50 micra in thickness were made within 2 to 3 hours after removal. These were kept in normal saline until used. Prior to staining with FSA the sections were placed in a 1 per cent solution of mercuric chloride for 5 minutes, following which they were stained for 15 minutes in the FSA solution. The stained sections were kept in a solution of 0 01 normal HCl containing 1 per cent sodium bisulfite. Immediately after the washing the sections were examined grossly and through the microscope. A positive reaction for aldehydes was indicated by a purple color. When sections were treated otherwise than as indicated above control sections from the same organ were run in parallel to the experimental strips except for the special procedure under consideration. The purplish blue color which developed in the control sections by means of FSA was designated at 4 plus and the color of the experimental sections compared to it. In this way a rough estimate could be made of the depth of color involved in each experiment. When kept in watery solution of SO$_2$ the stained sections retained their color for several days. Permanent preparations of these stained sections cannot be obtained because the aldehyde fuchsine compound decomposes into acetaldehyde bisulfite and fuchsine sulfurous acid with decolorization of the sections.

RESULTS A The staining reaction of tissue aldehydes with fuchsin sulfurous acid solution Sections of liver and kidney from 25 rats, 10 guinea pigs, 10 rabbits, 10 cats and 4 dogs were examined. In the liver, only the portal vessels and the central veins stained purple with FSA. The parenchyma did not stain.

The kidney stained deeply, although the stain was not distributed uniformly. The glomeruli stained lightly but definitely and consistently. Only isolated tubules stained in the outer cortical region. The deepest and most uniform staining was seen in the intermedullary zone and in the upper portion of the medulla. The pelvic part of the medulla remained almost unstained so that on gross examination there was always evident a sharp demarkation in staining depth between the cortical and the pelvic part of the medulla. Of all the species examined the rat kidney stained the least intensely. This may be connected with the fact that the rat kidney does not contain aminoxidase, Holtz, Heise, and Luedke (17)

When brought into a dilute solution of NaOH, the purple stain of the sections changed to a dull yellow. On re-acidification with HCl the original purple

stain reappeared. This is in contrast to the usual staining with fuchsin in which alkali causes the deep red color of fuchsin to appear. The fuchsin-aldehyde combination which is formed as a quinonoid dye is stable and becomes yellow at an alkaline pH.

B. *The effect of various experimental procedures on the plasmalogen content of the tissues.* The following experiments were performed in the hope of altering the renal function of the rat, in order to educe evidence of any correlation between renal activity and the distribution and depth of the FSA aldehyde stain in the kidney.

1. In order to increase the production of urine and thus influence the activity of the kidney, four rats were given 0.9 per cent saline as the sole source of fluid. At one and two week intervals on a voluntary saline intake there was increased diuresis of between 25 and 50 per cent. The distribution pattern of the plasmal in the kidney remained indistinguishable from that of the control rats drinking water.

2. Water was withheld for 5 days in a group of 4 rats during which period they lost 31 per cent of body weight. The kidneys lost proportionately in weight, yet no essential differences in the aldehyde staining pattern were apparent when compared to normal controls.

3. Two rats were subjected to unilateral nephrectomy, following which they drank less than normally. Ten to 14 days postoperatively, when the fluid intake had returned to the pre-operative level, the rats were sacrificed and the remaining kidney was tested for aldehydes with FSA. Despite the fact that this kidney was 25 per cent heavier than the kidney which had been removed, it showed no changes in the aldehyde-fuchsin color pattern.

4. The liver parenchyma did not react with FSA, showing that the liver cells do not contain an adequate concentration of aldehydes for the reaction. The absence of plasmalogen from the liver may be due to the presence of xanthine oxidase in this organ, rendering metabolic aldehydes unstable and therefore not easily stored. To test this hypothesis we performed experiments with xanthine oxidase obtained from milk.[3] The xanthine oxidase was allowed to act upon rat and rabbit kidney tissue aldehydes liberated from plasmalogen by prolonged incubation with dilute HCl. After incubation of the kidney sections in a solution of the enzyme for 18 hours, the sections no longer stained with FSA. The experiments were run at a pH between 6 and 7. Kidney sections incubated with non-enzyme containing solutions of similar pH lost some of their aldehyde content, but not so completely as after incubation with xanthine oxidase. The enzyme was shown to have no effect on the plasmalogen concentration. The absence of aldehyde from the liver may be due to the destruction of aldehydes by xanthine oxidase as soon as they are formed and before they can be converted to plasmalogen.

C. *The tissue aldehyde-binding power for certain amines.* Oster and Schlossmann (3) and Oster (18) showed that sections of guinea pig kidney which were incubated with p-aminobenzoic acid no longer stained with FSA. The solutions

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[3] Through the courtesy of D. E. Green, Department of Biochemistry, Columbia University, College of Physicians and Surgeons.

of p aminobenzoic acid were of sufficient acidity to decompose the plasmalogen so that the liberated aldehydes could combine with the amine Since amines do not combine with plasmalogen it is important to convert it to the free aldehyde when testing for aldehyde combinations with the amines Oster and Schlossmann (3) failed to obtain destaining of kidney sections by means of solutions of sulfanilamide This was because in neutral solution the plasmalogen was not decomposed to plasmal However, the present report shows that when the plasmalogen was first decomposed to plasmal by means of acid, sulfanilamide was capable of combining with the free aldehydes

The kidney of the rat, guinea pig, rabbit, cat and dog was chosen as the test organ in the experiments which follow because it is rich in plasmalogen and because of the ease of sectioning in the frozen state The following technic was used Frozen sections of the kidney were made as already described Several sections were stained immediately with FSA and the color recorded for depth from plus to 4 plus The remaining sections were incubated for 18 hours at 37°C in a solution of 0 9 per cent NaCl containing N/20 HCl, in order to liberate the aldehydes from the plasmalogen linkage The aldehyde containing sections were then washed in normal saline for 2 hours and transferred as needed to 10 cc portions of M/15 KH$_2$PO$_4$ solution containing 10 mg of the amine to be tested The pH was determined by means of the glass electrode and if necessary adjusted to a value of between 3 and 5 by the addition of ascorbic acid The ascorbic acid was found to have no influence on the development of the stain The aldehyde-amine combinations were incubated for 18 hours at 37°C , and the pH was again determined to insure that the solutions had remained below pH 5 The sections were then stained with FSA The depth and distribution of the color which developed was compared with that of the control sections which had been incubated in parallel at a pH below 5 but without the addition of any chemicals to the buffer The depth of stain of each control section was assigned an arbitrary value of 4 plus and the color of amine incubated sections referred to it from 0 to 4 plus The results are reported in the appended table

At a pH of 7 5 the liberated aldehydes disappeared spontaneously, and their fate was unknown Apparently they did not revert to the original plasmalogen linkage because incubation with HgCl$_2$ or N/100 solutions of HCl did not restore the original aldehyde content However, after a short incubation in a N/1 solution of HCl at 60°C the sections became stained Incubation of sections from the same kidney in buffer solutions of phosphate, bicarbonate or serum having a pH of 7 to 7 5 resulted in the disappearance of the aldehydes It was concluded that the influence which caused this change was the pH, and not a more specific action of some particular buffer ion

Table 1 summarizes the results obtained with various amines The results listed were obtained on the kidneys of 15 rats, 8 guinea pigs, 8 rabbits, 10 cats and 4 dogs The experiment was performed on sections of kidneys taken from 3 different animals except for the dog where only 2 were used In some cases the amine compounds themselves stained the sections from light to dark brown, but only in the case of o aminophenol, which stained the tissue dark brown was

there any serious interference in the determination of the depth of the purple color of the aldehyde reaction. Some of the compounds did not dissolve com-

TABLE 1

Depth of stain of kidney tissue with FSA after hydrolysis with 0 05N HCl for 18 hours at 37°C and subsequent incubation with each drug for 18 hours (pH 3 to 5)

NO	COMPOUND TESTED, 0 1 PER CENT	SPECIES OF ANIMAL					NO
		Rat	Guinea pig	Rabbit	Cat	Dog	
1	Solution at pH 4 3	++++	++++	++++	++++	++++	1
2	Solution at pH 7 4	±	±	±	±	±	2
3	Aniline hydrochloride	−	−	−	−	−	3
4	o Aminophenol	−	−	−	−	−	4
5	m-Aminophenol	−	−	−	−	−	5
6	p-Aminophenol	−	−	−	−	−	6
7	Acetanilid	++++	++++	++++	++++	++++	7
8	p-Aminoacetanilid	−	−	−	−	−	8
9	p-Toluidine	−	−	−	−	−	9
10	o-Toluidine	−	−	−	−	−	10
11	m Toluidine	−	−	−	−	−	11
12	o Nitroaniline	++++	++++	++++	++++	++++	12
13	m-Nitroaniline	−	+	±	±	±	13
14	p Nitroaniline	+++	++	++	+++	++	14
15	p-Aminobenzoic acid	−	−	−	−	−	15
16	Sulfanilic acid	−	−	−	±	−	16
17	Arsanilic acid	−	−	−	+++	−	17
18	Anthranilic acid	−	±	−	±	−	18
19	Mapharsen	−	−	−	±	±	19
20	Sulfanilamide	−	−	−	++	±	20
21	Sulfathiazole	±	−	−	++	+	21
22	Sulfapyridine	+	+	+	+++	++	22
23	2 Aminopyridine	++++	++++	++++	++++	++++	23
24	Procaine hydrochloride	±	+	+	++++	+	24
25	Benzylamine	+++	+++	++++	++++	++++	25
26	d-l, alpha Phenylethylamine	+++	+++	++++	++++	++++	26
27	d-l, beta-Phenylethylamine	−	−	−	−	−	27
28	Tyramine hydrochloride	++++	++++	++++	++++	++++	28
29	Amphetamine sulfate	++++	++++	++++	++++	++++	29
30	Ephedrine sulfate	++++	++++	++++	++++	++++	30
31	Epinephrine tartrate	++++	++++	++++	++++	++++	31
32	Naphthylamine	−	−	−	−	−	32
33	Isoamylamine	++++	++++	++++	++++	++++	33
34	Histamine phosphate	++++	++++	++++	++++	++++	34
35	Benzidine	−	−	−	±	−	35
36	Urea	++++	++++	++++	++++	++++	36
37	Phenylurea	++++	++++	++++	++++	++++	37
38	Amino acids	++++	++++	++++	++++	++++	38
39	Ascorbic acid	++++	++++	++++	++++	++++	39

pletely in the buffer solution In spite of that they combined readily with the tissue aldehydes. None of the compounds tested gave color reactions with FSA or interfered with the aldehyde color once it was established.

Close scrutiny of the table reveals the following facts:

a. Compounds with a primary amino group on the benzene ring combined with the tissue aldehydes as judged by the lack of staining with FSA. These include the stereoisomers of aminophenol, of aminobenzoic acid, and of toluidine. The stereoisomers of nitro-aniline did not behave uniformly. Ortho- and para-nitro-aniline failed to fix the aldehydes while with meta-nitro-aniline no staining was obtained.

b. Benzyl compounds with an amino group on a side chain, aliphatic amines and aliphatic amides did not form combinations with the tissue aldehydes which were stable under the influence of the acidity of the FSA reagent, with one exception. Beta-phenylethylamine fixed the tissue aldehydes so that they no longer stained with FSA, while the alpha-phenylethylamine homologue did not.

c. Incubation with acetanilid which has a secondary amino group, did not prevent staining with FSA.

d. A mixture of amino acids obtained from a casein hydrolysate[4] when incubated with the sections did not abolish the staining reaction with FSA.

e. Miscellaneous compounds such as histamine and 2-aminopyridine did not influence the staining of the tissue sections.

In general, the tissues of the 5 species of animals which were examined reacted similarly to incubation with the various amines listed in the table. There were slight (1 plus) quantitative differences in the staining but these were considered as insignificant. The cat kidney stained differently than did the kidneys of the other species, to arsanilic acid and to procaine hydrochloride. The reason for these differences is not clear but it may be that the cat kidney contains aldehydes which differ chemically from aldehydes found in the kidneys of other animals.

DISCUSSION. The failure of tissue aldehydes to stain with FSA following incubation with certain amines may be explained by the formation of Schiff's bases. In order that the aldehydes may react with FSA it is necessary to liberate them from their "plasmalogen" linkage, in which form they occur in the tissues. One of these linkages is an acetal (5) and most of the aldehydes are of the higher fatty series (4). If it is assumed that at any given moment in the metabolism of acids aldehydes are formed then these aldehydes could combine with endogenous or administered amines by forming Schiff's bases. In this way cellular metabolism would be deranged and the pharmacological effect of the specific drug would become manifest. The aldehyde-amine reaction was carried on at a pH of from 2 to 5, because the acid or $HgCl_2$ liberated aldehydes disappear spontaneously upon incubation at a pH above 7. This does not mean that the Schiff's base does not take place at a physiologic pH, for one of the mechanisms for the disappearance of aldehydes at a neutral pH may be the formation of a Schiff base between the tissue aldehydes and endogenous amines. Indeed Schoepf et al. (18) have shown that the rate of formation of a Schiff's base between dihydroxyphenylethylamine and dihydroxyphenylacetaldehyde is increased markedly as the pH increased from acid to neutral. One

[4] Mead and Johnson.

possibility for the antiseptic action of the sulfonamides is the formation of a Schiff's base with the aldehydes of the bacteria, thus interfering with the adequate metabolism of fats. This hypothesis received support from the well known antagonism between p-amino benzoic acid and the antiseptic action of the sulfonamides (19). It seemed possible that the sulfonamides (or other amines) should compete with p-aminobenzoic acid for some "acceptor" substance and that this acceptor substance should be an aldehyde. The conversion of aldehyde to pasmalogen by the normal cell may be a protective mechanism against the formation of Schiff's base with amines and this may not be the case for certain bacteria.

SUMMARY

1. The staining reaction for aldehydes with fuchsin sulfurous acid is described for liver and kidney tissues of 5 species of animals.

2. The formation of condensation products (Schiff's base) between tissue aldehydes and various amines and the influence of hydrogen ion concentration is described.

3. The significance of the formation of Schiff's base as a detoxification mechanism and the interference of p-amino benzoic acid with the antiseptic powers of the sulfonamides is discussed.

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CHRONIC AND DELAYED TOXIC EFFECTS OF CERTAIN SATURATED AND UNSATURATED HALOGENATED HYDROCARBONS IN WHITE RATS AND WHITE MICE

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The comparative anesthetic potency and acute toxicity of a series of saturated and unsaturated mono halogenated hydrocarbons have been the subject of an extensive investigation (1–8) *In vitro* chemical studies (9 13) and *in vivo* hydrolysis experiments (14) indicate that the mono halogenated olefins are more stable than the mono halogenated paraffins Since it seemed improbable that tissue damage is caused by the *in vivo* liberation of the acid halide in the case of either the saturated or unsaturated compounds (14), chronic and delayed toxicity studies were instituted on a series of saturated and unsaturated mono halogenated hydrocarbons and some of their more probable hydrolytic products

Simple hydrolysis of the 2 types of compounds could conceivably proceed by the following pathways

a) *Saturated compounds*
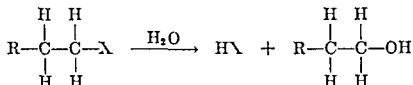
b) *Unsaturated compounds*

1 halo
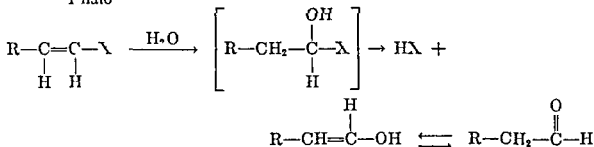
2 halo
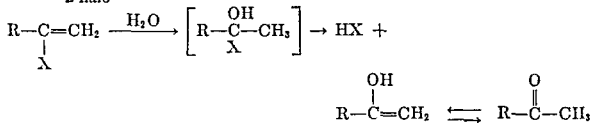
X = halogen atom (chlorine or bromine)
R = carbon chain

[1] Now at Baylor University College of Medicine

It has been suggested (8) that the enol form of ketones and aldehydes might be sufficiently reactive at sites of production in the body so that combination with enzymes or other reactive molecules might result in tissue damage.

EXPERIMENTAL   Three types of experiments were performed, viz., rat inhalation, rat injection and mouse inhalation.

Rat inhalation   Four adult white rats (Sprague-Dawley Strain) were exposed by the Fuhner method (15) 30 minutes daily for one week, to anesthetic vapor concentrations in oxygen which had previously been shown to be anesthetic for white mice (2) (see table 1). At the end of the week, 8 hours after the last anesthetic administration, all animals were killed *vis ad capitem*, and samples of the liver and lungs removed for histo-pathologic examination

Rat injection   Five adult rats (Sprague-Dawley Strain) were injected subcutaneously on a per kilo body weight basis with varying amounts of the agents in olive oil (see table 1). The quantity of agent used was that which had previously been found to be anesthetic

TABLE 1

Compounds used in white rats

COMPOUND	INHALATION EXPOSURE CONCENTRATION	INJECTION SUBCUTANEOUS DOSAGE
	mM /l	*cc /kg in 0 5 cc /kg olive oil*
1 chloro propane	1.7	0.149
2 chloro propane	3.0	
1 chloro propene	2.2	0.183
2 chloro propene	2.5	0.209
1 bromo propane	0.8	0.072
2 bromo propane	1.4	
1 bromo propene	1.1	0.074
2 bromo propene	1 2	0.086
propionaldehyde		0.215
iso-propyl alcohol		0.233
n-propyl alcohol		0.230

in 1 l of oxygen when in vapor form   In addition 3 of the possible products of hydrolysis propionaldehyde, iso-propyl alcohol and n-propyl alcohol were dissolved in olive oil and injected subcutaneously in other animals   The quantity of each hydrolysis product to be injected was calculated from the maximum amount of each which could be released by complete hydrolysis of 3 mM of 2-chloro-propane, one of the less potent anesthetic agents of the group   Three rats were killed at the end of one week, 8–12 hours after the last injection   The remaining 2 received no further injections and were killed 1 week later Samples of liver were taken for histo-pathologic examination

Mouse inhalation   In the third type of experiment 2–4 white mice (mixed strains) were anesthetized once for 30 minutes, with the exception of those exposed to bromo-ethane which were anesthetized for 60 minutes   The compounds investigated and concentrations in mM /l of oxygen employed in this experiment were bromo-ethane, 0 75, bromo-ethene, 2 0, 1-bromo-propane, 0 5, 1-bromo-propene, 1 0, 1-bromo-2-methyl propane, 0 5, 1-bromo-2-methyl propene, 0 75, 1-bromo n-butane, 0 25 and 1-bromo-butene-1, 0 25 and 0 5   Samples of heart, liver and kidney were taken for histo-pathologic examination

Tissues were fixed in either formaldehyde 10% or in Helley's solution   Routine paraffin sections were stained with hematoxylin and eosin.

RESULTS. The incidence of histo-pathological findings for the inhalation experiments in white rats are indicated in figure 1 and for the injection experiments in figure 2
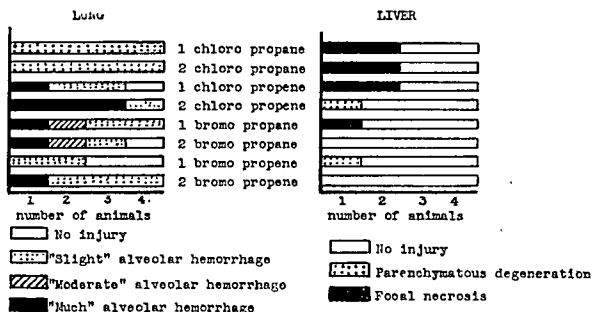
FIG. 1. INCIDENCE OF HISTOTOXIC EFFECTS IN WHITE RATS DUE TO DAILY INHALATION OF CERTAIN HALOGENATED HYDROCARBONS FOR ONE WEEK
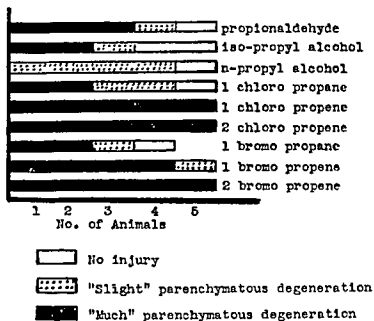
FIG. 2. INCIDENCE OF HEPATOTOXIC EFFECTS IN WHITE RATS INJECTED FOR ONE WEEK WITH HALOGENATED HYDROCARBONS AND CERTAIN HYDROLYSIS PRODUCTS

Rat inhalation experiments. All of the compounds produced some lung injury which was indicated by intra-alveolar hemorrhage. Only 2-chloro-propene, 1-bromo-propane and 2-bromo-propane produced "moderate" to "considerable" hemorrhage in 2 or 3 out of 4 rats; 1-chloro-propene and 2-bromo-propene were

definitely injurious in 1 out of 4 animals. Significant liver damage resulted from the action of 1- and 2-chloro-propane, 1-chloro-propene, and 1-bromo-propene in 1 or 2 out of 4 rats.

Rat injection experiments. In animals killed at the end of the first week, considerable parenchymatous degeneration was observed in the livers of all receiving: 1-chloro-propene, 2-chloro-propene and 2-bromo-propene, while it was present in 2 of 3 receiving 1-bromo-propene. Animals receiving the other compounds of the series either showed slight parenchymatous degeneration or no injury attributable to the agents. All of the animals which were allowed to survive two weeks showed slight to well established parenchymatous degeneration and one receiving n-propyl alcohol showed focal necrosis.

Mouse inhalation experiments. Marked histo-toxic effects were observable from all compounds studied. Advanced degenerative changes were present in the liver and kidneys of virtually all animals. At least one animal of each group showed marked and extensive necrosis of the liver. In addition, bromo-ethene and 1-bromo-propene produced widespread renal tubular necrosis. The myocardium of all animals showed parenchymatous degeneration.

DISCUSSION. With the small groups of animals employed it is difficult to make any definite biochemorphic generalizations with respect to tissue damaging properties. There is some indication from the rat inhalation experiments that unsaturation tends to facilitate irritation of lung epithelium. Observations (16) on dogs and rabbits which were anesthetized with the 1- and 2-bromo and 1- and 2-chloro-propenes support this finding in that there was excessive mucous secretion during the anesthetic period and in that respiratory difficulty was sometimes seen for a few hours after the anesthesia had been terminated. Other compounds in the series could be considered as having irritant potentialities if employed in concentrations approaching the lethal range. These findings confirm those of Marsh (8, 17) with respect to the lung injury which he observed in mice repeatedly anesthetized with 1 and 2-bromo-propene.

Inhalation experiments with rats indicate that the 1 and 2 chloro-propanes and 1-chloro-propene possess greater potentialities for producing hepatic injury than the brominated propanes or propenes. Since parenchymatous degeneration is indicative of only mild injury and is reversible, the results from the injection experiments might lead to the conclusion that these compounds are not significantly hepatotoxic. However, these animals, though they received daily injections of the total anesthetic amount of each agent, may not have absorbed sufficient quantities from the subcutaneously placed oil depot to cause hepatic injury. This would be in keeping with the findings that the pharmacological activity of fat soluble substances is reduced by dissolving them in oil (18). However, it does not necessarily follow that the chronic toxicity of a given agent would be reduced by this method of administration, unless the rate of detoxication exceeded the rate of absorption in this series, since some of the same compounds when administered by inhalation caused significant hepatic injury.

The delayed toxic effects observable in white mice killed 24 and 48 hours after single anesthetic periods of 30-60 minutes indicate that this species is more susceptible to the hepatotoxic action of these compounds than is the white rat. In addition, these agents are all renotoxic in the white mouse.

SUMMARY AND CONCLUSIONS

1. The 1- and 2-chloro-propenes and 1- and 2-bromo-propenes and 1-bromo-propane are markedly irritant to rat lung epithelium. The 1- and 2-chloro-propanes and 2-bromo-propane are slightly irritant when employed in anesthetic concentrations used in these experiments.

2. These anesthetic concentrations of 1-chloro-propane, 2-chloro-propane, 1-chloro-propene and 1-bromo-propane produced significant hepatic injury. None of the other compounds investigated were significantly hepatotoxic when administered by inhalation to white rats

3. In the subcutaneous dosages employed, none of the agents were significantly hepatotoxic to white rats. Reasons for the differences in results between inhalation and injection experiments are discussed.

4 Both the saturated and unsaturated mono-bromo compounds in the series bromo-ethane and ethene to 1-bromo-butene-1 and 1-bromo n-butane produced severe renal and hepatic injury in the white mouse

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BIOCHEMICAL ASPECTS OF THE TOXICITY OF ATABRINE

I. Acute Effects of Massive Doses in the Rat

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Additional investigations of the pharmacology and toxicology of atabrine are needed because of its widespread use and known accumulation in the animal organism (1, 2, 3). An objective method for determining, quantitatively, the onset and course of toxic reactions to the drug is highly desirable. Consequently, we have studied the effects produced by large oral doses of atabrine upon the gastro-intestinal tract and the liver of the rat in the hope of establishing a reflection of these effects in the blood chemistry data. Conditions of fasting and protein deprivation (4, 5) have been shown to augment the liver damage produced by chloroform, and repeated doses of atabrine are known to produce liver necrosis (6). It appeared that inanition might yield a similar increase in the damage produced by atabrine. The drug was, therefore, administered to animals in varying degrees of inanition as described in the experimental part.

EXPERIMENTAL. Albino rats of the Wistar strain, weighing between 150 and 250 grams, and maintained on a stock diet were used. A dose of atabrine equivalent to 50 per cent of the L.D. 50 (450 mgs. per kg. body weight) was given by stomach tube to 4 groups of rats in various states of nutrition as follows:

Group 1—50 rats, without prior fasting. Animals were then taken off diet. Rats were sacrificed 24 and 48 hours after dosing and blood samples were taken for analytical purposes. None of the animals died as a result of the administration of the drug.

Group 2—81 rats, after fasting over night. Eighteen, or 22 per cent, of the rats died an acute, convulsive death within 30 to 60 minutes after the administration of the drug. Surviving animals were continued off diet, and groups of rats were sacrificed 2, 6, 24 and 48 hours after dosing and blood samples were taken.

Group 3—75 rats, after fasting for 36 hours. Within 30 to 60 minutes, 36, or 48%, of the animals died acutely. Surviving animals, maintained off diet, were sacrificed for blood samples 2, 6, 24 and 48 hours after dosing.

Group 4—48 rats which had been maintained for several weeks on a low-protein diet[1] were dosed orally after a fast of 36 hours. Of these animals 28, or 58%, died acutely. Blood samples were taken from surviving animals 24 and 48 hours after dosing.

Group 5—To determine whether similar results were obtained at lower dose levels of the drug, a fifth group of stock animals was given 18 mgs. of atabrine per 100 grams body weight (20% of the L.D. 50) by stomach tube after an over-night fast. None of the animals died.

All animals, deprived of food throughout the test period, were sacrificed by decapitation and blood samples were collected. Oxalated plasma was used for the determination of bicarbonate (7), chloride (8), fibrinogen (9), and prothrombin time (10). Serum samples

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[1] This low-protein diet had the following composition: vitamin-low casein, 6; dextrose, 78; crisco, 8; corn oil, 1; dried, whole beef liver, 1; salt mixture, U.S.P. #11, 4; and cod liver oil, 2%. A supplement containing 80 mgs. each of thiamine, riboflavin and pyridoxine, 800 mgs. each of nicotinic acid and calcium pantothenate and 10 grams of choline chloride, all dissolved in 500 cc. of 20% alcohol was added to 10 kgm. of diet.

were analysed for total protein by the falling drop (11) and the micro Kjeldahl methods (12)   Determinations of bilirubin (13) and the icteric index (14) were also performed, but these fell within the normal range and are therefore omitted from subsequent tabulations   Urinary urobilin determinations were performed according to the method of Watson (15)   When no fecal contamination of the sample occurred, no increase in the urinary urobilin was observed   Values obtained are not recorded

The severe dehydration produced in these animals reduced the volume of blood obtained from each rat to 2 to 3 cc   Consequently it was not possible to perform all analyses on a single sample of blood   We have not pooled blood samples but have preferred to perform as many different determinations as possible on a single sample and have tried to use 5 or more samples for the determination of each blood constituent at each time interval but this was not possible in all cases   Control series of animals were sacrificed under conditions comparable to those imposed on the test animals

RESULTS

The deaths produced acutely in the animals included in the first four groups appear to be related to the dietary history of the animals   They cannot be explained simply by an increased rate of absorption of the drug since the stomach and small intestine of the rat are essentially cleared of food by an over night fast   Only 22 per cent of the animals were killed by the drug after an over night fast but 48 per cent died after a 36 hour fast, and 58 per cent of the low protein animals died after a 36 hour fast   While no significance is placed in differences of 10 per cent   the trend from 22 to 58 per cent appears real

At post mortem examination all animals showed markedly distended, fluid filled gastro intestinal tracts *   The distention appeared most marked in the rats sacrificed 6 and 24 hours after the administration of the drug, but the distention was severe in those animals sacrificed 2 and 48 hours after dosing   The contents of the small intestine appeared serous in nature and were tinged with blood in 5 to 10 per cent of the animals   All animals which survived for more than one hour after do ing showed evidences of diarrhea   All animals sacrificed 48 hours after dosing exhibited porphyrin encrusted whiskers   This may be taken to indicate dehydration (16)

Of the rats in group 5 namely, those animals receiving the smaller dose of the drug 10 rats sacrificed 2 hours after the administration of the drug showed a rather marked distention of the gastro intestinal tract   Seven of the 10 rats presented evidences of diarrhea and a marked congestion of the small intestine was noted in one rat   Similar but less marked signs were observed in the rats sacrificed at the 6 hour interval while those sacrificed 24 hours after the administration of the drug showed no signs of diarrhea or distention

The analytical data recorded in table 1 include only the values obtained in the determination of the blood bicarbonate, chloride and serum protein   These data indicate that a severe dehydration was produced within 2 hours after the oral administration of the atabrine   The serum protein values highest 2 hours after dosing begin to fall at 6 hours and tended to return to normal at 24 hours after dosing   A severe acidosis was also produced in these animals   The initial

* Large oral doses of the drug also produce severe distension of the intestinal tract of the rabbit

TABLE 1

Rat blood analyses

TIME AFTER DOSING	BICARBONATE (VOLS. %)				CHLORIDE (MG. % AS Cl)				SERUM PROTEIN (GRAMS %)		
	Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3
hrs.											
2		51.1	53.6	36.5		394	407	375		9.2	8.2
		50.4	40.8	30.3		384	397	365		8.2	8.0
		47.0	32.8	29.0		378	391	366		8.2	8.0
		35.8	31.0	28.6		372	378	360		7.8	7.8
		33.9				371					7.6
6		35.6	34.2			433	397			7.4	7.8
		34.8	32.8			427	397			7.2	7.4
		33.7	25.7			415	366			7.0	7.1
		32.4	25.7			392	353			6.8	6.8
		26.3				372				6.2	
						366					
						355					
24	61.9	55.8	54.8	46.4	379	353	373	354	7.4	7.1	6.7
	59.5	51.6	54.0	41.9	371	349	366	316	6.2	7.0	6.5
	58.4	51.2	45.5	38.2	368	347	364	305	6.2	6.8	6.3
	53.5	50.2			368	337			6.0	6.6	
	53.0	49.8			367	335			6.0	6.4	
	52.8				366	332				6.2	
	50.4				360					6.0	
	50.4				353						
	46.0										
Control data	50.8		53.9	45.3	387			375	6.0		5.9
	48.5		49.7	44.5	373			366	5.8		5.8
	46.4			38.9	370			354	5.6		5.7
					366						
48	53.4				359			354	6.4		
	52.3				357			348	6.4		
	50.9				354			348	6.0		
	50.0				348			342	5.9		
	49.3							334	5.8		
	49.2							323	5.5		
	46.5										
	46.2										
	42.0										
Control data	50.8				384			381	5.9		
	50.0				378			375	5.9		
	49.8				367			360	5.8		
	47.5				365			359	5.8		
								356			

hemoconcentration, resulting from the flow of fluids into the intestinal tract, was accompanied by loss of blood base. The plasma bicarbonate began to fall 2 hours after dosing. It reached a minimum at 6 hours and tended to return to normal 24 hours after the administration of the drug. The diarrhea, produced by the drug, appears to account for this loss of bicarbonate. Although the loss of base in watery stools is in excess of the chloride loss, there is usually a severe loss in blood chlorides as well. This is not evident in the plasma chloride values obtained at the 2 and 6 hour intervals because the hemoconcentration produced values that appear normal, or even elevated. The real chloride deficit tends to become apparent in the lowered values obtained 24 and 48 hours after the administration of the drug when the blood volumes returned to normal values. This delayed drop in plasma chloride is not a result of starvation.

TABLE 2

Blood analyses following the oral administration of 18 mgs. of atabrine per 100 gr. body weight to over night fasted rats

TIME AFTER DOSING	BICARBONATE	CHLORIDE	PROTEIN
hours	*vols %*	*mgs %*	*grams %*
2	37 5	394	7 9
	35 3	388	7 9
	31 7	397	7 8
	29 9	368	7 7
			7 5
6	49 4	384	7 4
	46 2	374	7 4
	46 1	374	7 2
	44 5	374	6 5
	39 7	371	6 2
24	46 9	352	6 6
	45 1	352	6 2
	44 1	350	6 2
	43 5	332	6 0

The control data included in table 1 indicate that starvation did not produce a bicarbonate deficit in the animals of groups 1, 2 and 3, but a deficit was produced in the animals of group 4, and upon administration of atabrine these animals developed a severe bicarbonate deficiency more rapidly and the mortality was greater than that observed in the animals of the first 3 groups.

The analytical data shown in table 2 were obtained with the animals of group 5, and are similar to those presented in table 1.

The tissues of the first four groups of animals were examined by Dr. Henry Siegel who will present, elsewhere, his findings on the pathology produced by atabrine. Evidences of severe liver necrosis were found in all of the animals receiving this large dose of the drug (50 per cent of the L D 50). In view of the

liver damage observed, it was of interest to determine if this damage was reflected in the chemical findings

Table 3 includes the results of the bromsulfalein (17), prothrombin, and prothrombin determinations. These determinations were performed 24 and 48 hours after the administration of the drug, at which time the blood electrolyte picture had returned essentially to normal levels Comparison with the control data indicates an increased retention of bromsulfalein in all test animals. The higher retention in the animals of groups 3 and 4, as compared with those in groups 1 and 2, indicates a greater impairment of liver function among the fasted

TABLE 3

Tests following a single dose (50% L D. 50) of atabrine in the rat

DAYS AFTER DOSING	BROMSULFALEIN (% RETENTION)				FIBRINOGEN (MGS %)				PROTHROMBIN TIME (SECONDS)			
	Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4
1	45	42	60	65	405	382	278	462	20 6	29 6	28 3	36
	37	37	55	55	338	386	244	380	17 7	22 6	27 4	26
	27	32	50	55	336	325		270	17 6	20 8		20 6
	27	30	47	55	314	310			16 6	20 2		
	25			45								
2	32	40	45	40	393	367	396	626	42 1	48 4	45 4	48 8
	30	30	42	37	322	390	320	416	34 8	27 0	26 4	40 0
	30	30	40	35	275	348			19 4	25 8	25 8	34 2
	30	30	35	35	165	319			17 7	17 8	23 7	30 4
	25			35							23 3	20 0
Control animals (not dosed) 1	17	16	20	20	219	172	193	332	16 0	20 0	21 8	15 8
	16	10	19	20	219	157	157	271	15 6	19 8	20 2	15 6
	15	12	15	17	172			203	14 7	19 2	19 8	15 4
	15		15	17	147			106	14 0		19 0	14.8
	13			15								
2	19	25	17	20	203	178	150	312	17 4	18 5	20 4	19 5
	17	18	16	17	192	164	145		15 0	17 0	20 1	18 8
	17	20	15	17	144	185				16 7	19 3	16 4
	15		13	15	114							
	13			12								

animals This result is in agreement with the extensive observations reported by Whipple (4), Ravdin (5), and their co-workers The prothrombin time in the test animals is somewhat greater than in the control animals, but the differences are not great and there seem to be no significant differences between the four groups of test animals Plasma fibrinogen values are increased in all test animals [3] Elevated fibrinogen values, which are usually associated with inflammation and tissue destruction, appear to be a sensitive indicator of atabrine toxicity and will be discussed more fully in the following paper

[3] Large single doses of the drug also produce increased plasma fibrinogen levels in the dog.

No attempt was made to correlate the above findings with blood concentrations of the drug since it was shown (18) that, at these high dose levels, the whole blood concentrations of "total" atabrine were notably constant and bore no apparent relationship to the dose administered   High concentrations of the drug were found, however, in the liver (19)   These observations, together with findings on the distribution and excretion of the drug suggested that the drug is deposited in the tissues and is slowly liberated, thereby maintaining a rather constant blood concentration of the drug and its metabolites (20)

SUMMARY AND CONCLUSIONS

The mortality of a single dose of atabrine (equivalent to 50% of the L D 50) was increased in the rat from 0 to 48% by causing the animals to fast for increasing lengths of time

This dose of the drug causes a severe irritation of the gastro intestinal tract resulting in a flow of fluid into the stomach and intestines   The distention is followed by diarrhea   There is a marked hemo concentration, a loss of blood chlorides and a severe loss of blood bicarbonates   Similar results were obtained with a smaller dose of the drug (20% of the L D 50)

The larger dose of the drug produced a severe liver necrosis   As judged by the retention of bromsulfalein and prolongation of the prothrombin time, inhibition of liver function was produced by the drug and this inhibition was increased by fasting   Plasma fibrinogen concentrations were increased appreciably by the administration of atabrine

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BIOCHEMICAL ASPECTS OF THE TOXICITY OF ATABRINE

II. The Influence of the Diet upon the Effects Produced by Repeated Doses of the Drug

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Continuing our investigations into the nature of the toxic reactions produced by atabrine we have studied the influence of the diet upon the effects produced by repeated doses of the drug.

PART I

EXPERIMENTS WITH RATS. Two groups of albino rats of the Wistar strain were used as follows: One group was maintained on a low-protein diet (Diet #A8) for seven weeks and the other group was maintained on a high-protein diet (#A30). When the weights of the animals in these two groups were widely different—100 to 160 grams for the low-protein group, and 200 to 300 grams for the high-protein group—the animals were subdivided into four groups. The low-protein animals were transferred to diets #37 and #39. Both of these diets were low in protein (4%) but diet #37 contained only 2% fat whereas diet #39 contained 47% fat. The high-protein animals were transferred to diets #38 and #40. Both of these diets were high in protein (45%) but diet #38 contained only 2% fat whereas diet #40 contained 47% fat (the composition of all the diets and supplements are shown in table 1).

After a two to three week period of equilibration on these diets, five animals from each group were sacrificed for control purposes. As additional controls, five animals, maintained throughout the experiment, were sacrificed at the end of the experiment. The remaining animals (about 15 in each group) were then given oral doses of atabrine (4.5 mgm. per 100 gr. body weight) by stomach tube each day, six days of each week. Three, seven and twenty-four days after the dosing was begun, five animals from each group were sacrificed.

Individual blood samples, taken when the animals were sacrificed, were analysed for fibrinogen (1), prothrombin (2), icteric index (3), bilirubin (4), and occasionally for serum protein (5), and plasma bicarbonate (6). Bromsulfalein tests (7) were also performed. Icteric index and bilirubin determinations were consistently negative and are therefore omitted from subsequent tabulations. In general, five rats were used from each group at each time interval and each analysis was performed on samples from individual rats.

RESULTS. The data, shown in table 2, indicate anomalous results with the bromsulfalein test. In the preceding paper it was shown that control rats maintained on the stock ration retained 12 to 25 per cent of the bromsulfalein under the test conditions. This is higher than the values previously reported (7). The control data included in the present table 2 average from 17 to 43 per cent. It appears from these latter results that bromsulfalein retention in the rat is influenced by the dietary history of the animal and although an increased retention of the dye was observed when rats were given single, large doses of the drug (see Part I), no significant increase or trend is apparent in the data obtained in the present, chronic experiments.

As noted in the preceding article, determinations of the icteric index and

bilirubin gave negative results   Nor does the prothrombin time appear to be a sensitive indicator of impaired liver function since only after prolonged administration of the drug did the prothrombin time tend to be increased   These slightly elevated values were observed only among those animals maintained on diets #39 and 40   The plasma fibrinogen values however, were increased by as few as three daily doses   Following a drop in these levels, the plasma fibrinogen levels were increased as the animals on diets #37, #39, and #40 were continued on test   This was not equally true of the animals maintained on diet #38   In this group the fibrinogen values were increased in 2 animals after 3 doses of the drug, but in all the rest of the animals the levels were not greatly elevated as compared to the control values nor did the fibrinogen levels increase significantly as the animals were continued on test

TABLE 1

Diets used

	RAT DIETS*						DOG DIET† LOW PROTEIN
	A8	A30	37	38	39	40	
	%	%	%	%	%	%	
Casein (vitamin low)	6	30	4	45	4	45	3
Dextrose	78	54	83	47	43	2	66
Crisco	8	8	0	0	45	45	25
Corn oil	1	1	2	2	2	2	
Dried liver (whole beef)	1	1	2	2	2	2	
Salt mixture #11	4	4	4	4	4	4	4
							Bone ash
Cod liver oil	2	2					2

* Supplement #11 containing 80 mgm each of thiamine riboflavin and pyridoxine, 800 mgm each of nicotinic acid and calcium pantothenate and 10 grams of choline dissolved in 500 cc of 50% alcohol was added to 10 kgm of each diet used   Animals maintained on diets 37 38 39 and 40 were each given a drop of percomorph per week

† Addendum #31—10 mgm each of B_1 B_2 B_6 and 1 gram each of nicotinamide inositol calcium pantothenate and 10 gr choline chloride dissolved in 500 cc of 50% ethyl alcohol added to 10 kgm of diet   0 5 cc percomorph two times weekly

As judged by body weight, activity and condition of the pelage all control animals appeared in good condition although the pelage of those animals maintained on the high fat diets was somewhat greasy in appearance   Diet #39 was ketogenic and diet #40 was slightly so   Those animals which received the drug and were maintained on diets #37, 39 and 40 lost in weight as the test continued   Their activity was depressed and the pelage became disheveled   The animals presented evidences of diarrhea and the whiskers became encrusted with porphyrin like materials   In contrast to these rats, the test animals on diet #38 (high protein low fat diet) maintained their weight although they did not gain   Their activity and the condition of their coats were good   There were no evidences of diarrhea or porphyrin encrustations of the whiskers

It would seem from this experiment that these toxic effects of atabrine are

TABLE 2

Data obtained in chronic experiments in rats

DIET NO.	DATE	BODY WEIGHT gr.	FIBRINOGEN mgs. %	BROMSULFALEIN RETENTION %	PROTHROMBIN TIME sec.	BICARBONATE vols. %	SERUM PROTEINS gr. %
37	Control 9/29	141 (105–167)	170 (125–208)	30 (22–37)	16.5 (15.5–18)		
	10/2	170 (162–160)	248 (200–294)	37 (35–40)	19.3 (18.8–20)		
	10/6	155 (147–168)	231 (213–297)	35 (32–40)	18.6 (18.2–19.2)		
	10/23	124 (121–128)	450 (420–470)	43 (40–15)	18.6 (18.0–19.6)		
	Control 10/23	171 (135–201)	240 (204–295)	38 (35–40)	20.2 (18.0–21.8)		
38	Control 9/29	234 (188–267)	195 (178–214)	36 (35–40)			
	10/2	224 (198–244)	319 (255–414)	30 (27–32)	18.4 (17.0–19.8)		
	10/6	248 (233–265)	216 (161–280)	28 (25–35)	20.5 (19.2–22.4)		
	10/23	222 (207–233)	263 (225–295)	25 (20–27)	20.5 (19.4–21.2)		
	Control 10/23	270 (247–306)	209 (196–226)	17 (16–20)	21.0 (20.4–22.0)		
39	Control 9/29	233 (212–251)	121 (79–163)	32 (27–40)	18.4		
	10/2	260 (232–312)	283 (248–356)	35 (30–37)	18.3 (17.6–19.4)		
	10/6	246 (189–302)	232 (185–260)	30 (22–40)	19.7 (17.6–22.0)		
	10/26	188 (157–221)	318 (280–355)	34 (30–45)	22.5 (19.5–25.4)	30.9 (28.4–33.0)	5.1 (4.8–5.4)
	Control 10/26	211 (188–232)	125 (81–163)	35 (27–40)	19.8 (17.6–23.8)	39.0 (37.4–41.5)	5.8 (5.7–5.9)
40	Control 9/29	292 (258–318)	154 (128–203)	29 (22–35)	19.9 (18.8–20.6)		
	10/2	281 (262–326)	219 (210–230)	25 (20–30)	18.4 (17.8–18.8)		
	10/6	243 (185–234)	209 (144–243)	30 (27–35)	20.3 (19.4–21.2)		
	10/26	219 (175–295)	304 (173–424)	25 (17–35)	25.6 (20.4–34.2)	39.4 (36.6–42.0)	5.5 (5.1–6.1)
	Control 10/26	297 (232–389)	159 (132–179)	20 (12–32)	19.1 (17.8–20.4)	48.3 (44.7–50.4)	5.9 (5.1–6.2)

The figures given are averages. Those in parentheses give the range of variation.

largely prevented by the high protein diet, and further, that plasma fibrinogen values parallel the toxic effects of atabrine upon the liver[1]

PART II

EXPERIMENTS WITH DOGS A Dogs numbered 241 and 255 were given 50 mgm of the drug per kgm body weight per day six days of each week while dogs number 287 and 273 and 275 were given 25 10 and 5 mgm doses respectively These animals were maintained on stock rations Fasting blood samples drawn from time to time were used for analytical purposes The determination of the prothrombin time (2) blood sugar (8) icteric index (3) and bilirubin (4) and the urinary urobilin output (9) showed nothing of interest at the times when they were performed These data are consequently omitted from subsequent tabulations The data recorded in table 3 are concerned only with the determinations of serum protein (5) fibrinogen (1) albumin globulin (10) non protein nitrogen (11) urea (12) and cholesterol (13)

RESULTS Dogs numbered 241, 255 and 287, namely those animals receiving the larger daily doses of the drug underwent a loss in body weight and this loss in weight was accompanied by a diminished food intake The resultant mani tion, produced presumably by the anorexia vomiting and diarrhea induced by the drug is reflected in the decreased serum proteins, and more specifically in the albumin fraction of the serum protein, and in the diminished urea non protein nitrogen and cholesterol values obtained (table 3)

As in the acute experiments with rats described in the preceding paper and as in the chronic experiments with rats described above, plasma fibrinogen values were increased by the administration of atabrine to these three dogs It may be noted that dogs numbered 241 and 287 tended to show a return toward normal fibrinogen levels as the animals continued on test Dogs 273 and 275 which received 10 and 5 mgm per kgm doses of the drug respectively did not show increased fibrinogen values Neither was there a loss in body weight nor a diminution of the blood protein cholesterol urea or non protein nitrogen even though these dogs were continued on test for much longer periods of time than the three dogs receiving the higher dose levels of the drug

Since Whipple and his co workers (14) demonstrated the hepatic origin of fibrinogen numerous attempts have been made to use the determination of fibrinogen as a test of liver function In frank cases of liver degeneration such as occur in acute yellow atrophy, chloroform and phosphorus poisoning fibrinogen values are invariably lowered This is the opposite of the upward trend in the fibrinogen values observed by us Foster and Whipple (15) have pointed out however, that most infectious diseases traumatic injuries, and other conditions that produce destruction or inflammation stimulate fibrinogen production and that plasma fibrinogen levels may be increased in the presence of liver damage In this connection, Foster and Whipple (15) have pointed out that the actual destruction of liver tissue may cause increased fibrinogen values, and low values are obtained only when the loss of liver tissue has become sufficiently great that the organ can no longer meet the needs of the body for fibrinogen production

[1] The tissues of all animals were examined by Dr Henry Siegel who will present else where his findings on the pathology produced by atabrine

TABLE 3

Blood chemistry data obtained with dogs maintained on stock rations

DATE	BODY WEIGHT	SERUM PROTEIN	ALBUMIN	GLOBULIN	A/G RATIO	FIBRIN-OGEN	TOTAL CHOLES-TEROL	UREA N	N.P.N.
Dog No. 241. 50 mgm./kgm. Dosing begun 10/6/42. Body wt. 19.5 kg.									
	kgm.								
12/16/42		4.8	3.2	1.6	2.0	380		19	28
12/22/42	16.0	5.2	3.6	1.6	2.3	370	90		33
1/ 5/43	17.0	4.9	3.0	1.9	1.6	410	130		37
2/ 1/43	18.4	4.7	2.1	2.6	0.8	482	84	4	22
3/12/43	16.3	5.4	2.8	2.6	1.0	440			
3/30/43	16.0	5.4	3.2	2.2	1.5	358	68		6
Dog no. 255. 50 mgm./kgm. Dosing begun 9/19/42. Body wt. 9.0 kg.									
10/ 1/42	7.8	4.8				309	172		
10/29/42	8.7	4.9				396	106		
11/12/42	8.3	5.6				530	75		
Dog no. 287. 25 mgm./kgm. Dosing begun 1/25/43. Body wt. 8.65 kg.									
1/14/43	8.65	6.1	3.9	2.2	1.8	250	136		
1/29/43						390			34
2/20/43	8.0	5.3	3.3	2.0	1.65	332			
3/ 2/43	8.0	5.2	3.6	1.6	2.25	345	119	14	32
3/15/43	7.65	5.3				340			20
3/24/43	7.3	4.9	3.3	1.6	2.1	322	83		13
Dog no. 273. 10 mgm./kgm. Dosing begun 9/17/42. Body wt. 8.5 kg.									
10/ 1/42	8.4					314	150		
10/29/42	8.8	6.0				276	150		
11/12/42	9.1	5.8				175	110	9.5	27
12/ 3/42	9.1	5.8	3.5	2.3	1.5	239			27
12/22/42	9.7	5.4					98		
1/ 5/43	9.7	5.4				256	146	10	34
1/25/43	9.9	6.0	4.2	1.8	2.3	260	162	14	41
Dog no. 275. 5 mgm./kgm. Dosing begun 9/17/42. Body wt. 7.3 kg.									
10/ 1/42	8.1					200	110		
10/29/42	9.1	6.2				277	168		
11/12/42	9.3	6.1				241	123	20	32
12/ 3/42	9.4	6.4	4.1	2.3	1.8	210			31
3/12/43	9.4	5.3	3.1	2.2	1.4			17	41

In 28 determinations on 12 normal dogs the average fibrinogen concentration was 255 (±35, maximum deviation, 80) mgs. per cent. Serum protein albumin and globulin are given in grams %. Fibrinogen, cholesterol, urea N and N.P.N. are given in mgs. %.

It appears that the increased fibrinogen values observed following the administration of atabrine result from the toxic effect of the drug upon the liver

B Previous experiments indicate that the diet influences the toxicity of atabrine and the above experiments with dogs indicate that the drug, at least at the higher dose levels, produces an inanition It thus appears that a cyclical process occurs whereby the drug produces an inanition and as a result of this inanition, the toxicity of the drug is augmented Rather than to maintain dogs on different diets to investigate this phenomenon, it was decided to subject animals to a severe protein depletion and then to administer the drug chronically If the depletion influences the toxicity, signs and symptoms should appear in a shorter time among the depleted animals than among stock animals Further, toxic effects might then be reversed by the administration of liberal amounts of protein The following experiments were designed to test these assumptions

A group of dogs were placed on the low protein diet (table 1) on 6/9 Three months later the condition of the dogs was critical Two of the dogs died following perforation of gastric ulcers and the remaining six dogs were anemic and severely depleted in protein In order to keep the dogs alive they were permitted free access to lean meat on 9/29, 9/30 10/9 and 10/23 but otherwise the animals were continued on the low protein diet Dogs numbered 219 and 238 were reserved as controls and dogs numbered 222 227, 228 and 229 were given, by stomach tube 10 mgm of atabrine per kgm body weight for six days of each week beginning on test 10/7

Samples of blood were drawn at various times (indicated in table 4) These were analysed for fibrinogen (1) serum protein (5), albumin globulin (10), urea (12), non protein nitrogen (11), glucose (8) total cholesterol (13), bicarbonate (6) icterus (3) and chloride (16) Hematocrit values (17) prothrombin time (2) and Hanger Flocculation rates[*] (18) were also determined Due to limitations in the size of the blood samples it was not possible to perform all tests on each sample of blood Consequently, groups of analyses were performed on alternate samples of blood and when tests gave values which continued to fall within the normal range these tests were discontinued For example, determinations of bilirubin icterus prothrombin time, urinary urobilin, and the occasional brom sulfalein tests that were performed showed little of interest even though atabrine administration (19) and low protein diets (20) are known to produce liver damage

RESULTS As shown in the first three sets of analytical data (10/22, 11/2, 11/11) in table 4, the animals were depleted of protein serum protein ranged between 4 0 and 5 2 grams per cent After as little as 13 doses of the drug, fibrinogen values were increased and after 31 doses values as high as 444 mgm per cent were obtained among the test animals while the control animals #219 and 238, did not give values exceeding 264 mgm per cent During this time interval, when the animals were being maintained on the low protein diet, body weights remained relatively constant and as a result of the low protein diet blood urea and non protein nitrogen were low The cholesterol levels were somewhat elevated in these dogs While in most conditions of marked cachexia hypocholesterolemia occurs, starvation causes a hyperlipemia that affects all the lipoid constituents of the blood The hypercholesterolemia in these animals presumably resulted from starvation

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[*] The Hanger flocculation test is apparently valueless in dogs since in our hands all dogs regularly give four plus values

TABLE 4

DATE AND DIET	DOG NO.	WEIGHT	FIBRIN-OGEN	SERUM PROTEIN	ALBU-MIN	GLOBU-LIN	A/G RATIO	UREA N	N.P.N.	UREA/N.P.N.	HEMA-TOCRIT	TOTAL CHOLES-TEROL
1943												
10/22	219		167	4.0								289
13 doses	238		202	4.3								333
	222	5.9	286	4.5								250
	227	4.9	256	4.2								333
	228	6.2	342	5.2								272
	229	6.7	378	4.4			·					312
11/2	219	4.9	257	4.2				3.8	12.3	.31	28.4	226
23 doses	238	8.5	204	4.4				3.8	14.1	.27		271
	222	5.8	301	4.9				9.4	18.7	.50	37.5	298
	227	4.8	390	4.5				2.9	9.6	.30	29.5	306
	228	6.5	426	5.4				2.6	10.8	.24	29.0	395
	229	6.8	310	4.8				2.3	12.3	.19	29.0	397
11/11	219		264					8.3	16.6	.50	28.0	193
31 doses	238		250					8.2	15.7	.52	36.0	255
	222	6.2	355					11.5	23.3	.49	36.0	202
	227	5.1	444					7.2	11.9	.60	27.0	238
	228	6.8	362					5.5	12.1	.45	30.0	298
	229	6.8	367					6.5	13.6	.48	30.0	248
11/23	219	5.6	438	5.2				11.0	25.0	.44		230
	238	9.9	416	4.6				25.0	41.0	.61		196
	222	6.8	480					14.0	28.0	.50		170
	227	6.4	652	4.7				21.0	33.0	.64		194
	228	8.4	423	6.2				11.0	26.0	.42		194
	229	7.4	487	5.3				7.0	24.0	.29		201
11/30	219		435	5.5	3.1	2.4	1.3					
	238		355	5.9	3.3	2.6	1.3					
	222	6.9	653	5.8	3.1	2.7	1.1					
	227	8.0	433	5.3	3.0	2.3	1.3					
	228	10.0	355	6.5	3.5	3.0	1.2					
	229	9.4	503	6.3	3.0	3.3	0.9					
12/7	219		241	5.6	3.4	2.2	1.5	11.0	38.0	.29	37.0	
	238		238	6.0	3.5	2.5	1.4	15.0	45.0	.33	40.0	
	222	7.5	316	6.5	3.5	3.0	1.2	16.0	44.0	.36	37.0	
	227	8.9	294	5.9	3.3	2.6	1.3	12.0	34.0	.35	28.0	
	228	10.7	222	6.6	3.9	2.7	1.4	12.0	35.0	.33	32.0	
	229	10.2	275	6.0	3.2	2.8	1.1	10.0	29.0	.35	33.0	
1943 1/11	219			5.6	3.4	2.2	1.5	12.0	28.0	.43	38.0	142
	238		257	5.4	3.5	1.9	1.8	14.0	37.0	.38	44.0	131
	222	8.1	300	6.2	3.7	2.5	1.5	14.0	33.0	.43	40.0	134
	227	11.3	331	6.1	3.6	2.5	1.4	12.0	33.0	.36	36.0	148
	228	13.0	306	6.4	3.9	2.5	1.6	15.0	32.0	.47	35.0	188
	229	12.9	286	6.0	3.4	2.6	1.3	14.0	30.0	.47	32.0	159

TABLE 4—*Continued*

DATE AND DIET	DOG NO	WEIGHT	FIBRIN OGEN	SERUM PROTEIN	ALBU MIN	GLOBU LIN	A/G RATIO	UREA N	N P.N	UREA/ N P.N	HEMA TOCRIT	TOTAL CHOLES TEROL
1943												
2/2	219		205	5 0	3 4	2 5	1 4		23 0		39 0	146
	238		260	5 6	3 5	2 1	1 7		33 0		44 0	121
	222	7 7	310	5 7	3 5	2 2	1 6		31 0		41 0	124
	227	11 1	271	6 1	3 4	2 7	1 3		32 0		39 0	129
	228	11 5	221	6 1	3 7	2 4	1 5		32 0		39 0	193
	229	12 0	293	6 3	3 3	3 0	1 1		32 0		37 0	
3/3	219		272	5 6	3 7	1 9	1 9		32 0		40 0	150
	238		265	5 8	3 7	2 1	1 8		37 0		43 0	112
	222	8 6	296	6 0	3 7	2 3	1 6		31 0		41 0	118
	227	13 3	260	5 8	3 7	2 1	1 8		30 0		39 0	144
	228	14 1	172	5 9	3 9	2 0	1 9		26 0		40 0	193
	229	13 9	212	5 7	3 4	2 3	1 5				35 0	190
4/1	219	10 1	250	5 4	3 3	2 1	1 6	14 0	21 0		39 0	215
	238	16 7	195	5 2	3 3	1 9	1 7	11 0	34 0		41 0	139
	222	8 5	287	6 2	3 8	2 4	1 6	15 0	27 0		41 0	122
	227	13 5	203	5 8	3 8	2 0	1 9	13 0	29 0		39 0	116
	228	13 9	176	6 2	4 2	2 0	2 1	12 0	36 0		40 0	147
	229	13 4	276	5 9	3 5	2 4	1 5	14 0	29 0		37 0	124

Meat diet begun 11/16/42—meat discontinued 3/19/43

Serum protein albumin and globulin values are given in grams % fibrinogen urea N, N P N and cholesterol are given in gms % Hematocrit values are given in volumes per cent

A comparison of fibrinogen values obtained with these dogs maintained on the low protein diet (table 4), with the values obtained with dogs maintained on stock rations (table 3), shows a striking difference in the time required for the appearance of the increased fibrinogen values The protein deficient animals showed increased values within two weeks after administration of the drug was initiated Dog #273 which was maintained on a stock ration and received the same daily dose of the drug (10 mgm per kgm) did not show an increased fibrinogen level four months after initiation of drug administration At 25 mgm per kgm per day, dog #287 showed an augmented fibrinogen level one month after starting the drug and dogs #241 and 255 which received 50 mgm per kgm required two to four weeks of dosing before the levels were increased Thus, it appears that increased fibrinogen concentrations were induced by atabrine at an earlier date in protein depleted dogs than in dogs maintained on stock diet

In order to determine if the increase in the fibrinogen levels observed in the low protein dogs could subsequently be reversed, a diet relatively high in protein was administered This diet, composed of 1 part of Gaines food mixed with one part of horse meat, was given on 11/16/42 and daily thereafter As shown in table 4 the fibrinogen values were greatly increased in both the test and control animals for two weeks (11/23, 11/30) after restoring protein to the diet This non specific increase in the plasma fibrinogen may be related to liver damage

induced by the prolonged protein deprivation (20), but, nevertheless, one week later all these values fell, and remained at normal levels thereafter. It thus appears that the fibrinogen increase produced by the atabrine was reversed by the administration of protein.

Following restoration of dietary protein, the body weights of the animals increased even though the daily administration of the drug continued. The serum protein level increased slowly. Initially the globulin levels were a little high, but these underwent readjustment to normal levels in about one month's time. These observations are in agreement with the finding of Weech and Goettsch (21) who demonstrated that the administration of adequate diets after long periods of protein deprivation produces an immediate rise in globulin which may for a time be greater than the concomitant rise in albumin. The albumin fraction increased slowly. Weech (22) suggested that this delayed type of re-generation may result from injury to a mechanism, presumably in the liver, which is concerned with synthesizing albumin. In our dogs urea and non-protein nitrogen levels increased to normal values indicating no impairment of the kidneys' ability to eliminate nitrogenous waste products. In this connection it may be noted that we have examined many urine samples from all the dogs treated with atabrine in these studies and have found no evidence of albuminuria. The blood cholesterol levels returned to more normal values and did not appear to be greatly changed by the continued administration of the drug.

From the data recorded in tables 3 and 4 it is evident that fibrinogen values, which have been demonstrated to be increased by atabrine administration, are increased more rapidly in protein depleted dogs than in normal dogs, and as shown in table 4 this increase can be reversed by restoring protein to the diet of the depleted dogs.

SUMMARY

Rats maintained on a high protein-low fat diet appear to resist toxic effects upon the liver of the daily administration of atabrine to a greater degree than rats maintained on low-protein diets or a diet high in both protein and fat. Inhibition of liver function in the rat was not detected by the bromsulfalein test, the determination of the icteric index, bilirubin or prothrombin time. Plasma fibrinogen values appear to parallel the toxic effects of atabrine upon the liver.

Daily administration of large doses of atabrine (25 to 50 mgm. per kgm.) to dogs produces an inanition within 3 to 6 weeks. At lower dose levels (5 to 10 mgm. per kgm.) this is not evident three to five months after initiation of drug administration.

Plasma fibrinogen levels are more rapidly increased by the daily administration of atabrine in protein depleted dogs than in dogs maintained on a stock ration. This effect of the drug is reversed by restoring protein to the diet of the depleted dogs.

Determinations of the prothrombin time, icteric index, bilirubin, urinary urobilin, urea, non-protein nitrogen, urinary protein, and the Hanger flocculation time afforded no evidence of depressed function in these dogs.

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HYPOPROTHROMBINEMIA INDUCED BY ADMINISTRATION OF INDANDIONE DERIVATIVES

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A decrease or disappearance of blood prothrombin (1) may be produced by minute amounts of dicumarol (3,3'-methylene-bis-(4-hydroxycoumarin)). This compound acts as an anticoagulant only *in vivo* and, when given intravenously as well as orally, there is a latent period of 12 to 24 hours before the prolongation of prothrombin time becomes apparent. The effect appears to be highly specific, liver injury (2) and other toxic effects (3) resulting only from larger doses. The similarity of the hypoprothrombinemia induced by dicumarol and the effects of deficiency of vitamin K has suggested the possibility that dicumarol may act as a physiologically antagonistic chemical analogue of the vitamin (4). While vitamin K is relatively ineffective in counteracting the hypoprothrombinemia induced by dicumarol, large doses of the vitamin have been shown to decrease the effect produced by minimal doses of dicumarol (4). The production of thiamine deficiency disease by a pyridine analogue of thiamine and of a scurvy-like disease by glucoascorbic acid has been reported (5).

Hypoprothrombinemia may also be induced by compounds other than dicumarol. Lehmann (6) studied the effect of coumarin and dicoumarin derivatives on prothrombin time of rabbits. He found that dicumarol was 1,000 times as active as coumarin, 250 times as active as 4-hydroxycoumarin and 250 times as active as 3-methyl, 4-hydroxycoumarin. Acetylation of the hydroxyl groups of dicumarol or substitution of a methyl group for one of the hydrogens on the methylene group of dicumarol had little effect on its pharmacological activity. On the other hand, substitution of a carboxyl group for one of the hydrogens of the methylene group of dicumarol decreased the specific activity 80 times.

Salicylic acid, methyl salicylate and acetylsalicylic acid have been shown to produce a hypoprothrombinemia in animals (7) and man (8). The effective dose of salicylates is 25 to 100 times that of dicumarol and the change in plasma prothrombin is readily prevented by small doses of vitamin K. Link and his associates (7) observed that only those compounds show anticoagulant action which theoretically might yield an O-hydroxybenzoic acid derivative on degradation. Benzoic acid and m- or p-hydroxybenzoic acids had no effect on prothrombin time. Sulfaguanidine also results in a prolongation of prothrombin time (9) which is readily prevented by administration of vitamin K.

In the course of other investigations of the toxicity of various compounds in relation to their chemical structure, the observation was made that several indandione derivatives produced fatal hemorrhages and that the blood at death was incoaguable. A more intensive study of the effects of these compounds on

plasma prothrombin was therefore undertaken   These compounds have been shown to have a powerful and specific effect on depressing the prothrombin level in the blood, an action apparently identical with that of dicumarol   One of these compounds appears to be as potent as dicumarol

METHODS   The experiments were performed on albino rats and rabbits   Studies on acute and chronic toxicity were carried out by oral administration of single or repeated doses of the compounds dissolved in olive oil or by incorporating the compound in the diet

Blood for prothrombin determinations was drawn by cardiac puncture and the prothrombin time of undiluted oxalated plasma determined by a modification of Quick s method (10) using rabbit brain as the source of thromboplastin

The indandione compounds were obtained through the courtesy of Dr L B Kilgore (11)

OBSERVATIONS   (A) 2 isovaleryl 1,3 indandione
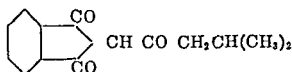
This compound has a pronounced effect in depressing the plasma prothrombin   In rabbits, the minimal effective oral dose is 5 0 to 7 5 mg per kg   The effect on prothrombin time with various doses in rabbits is shown in table 1   The time relations of change of prothrombin time following administration of this compound are shown in figure 1   The prothrombin time shows a rise at 24 hours but usually does not reach a maximum until 48–72 hours   Blood samples taken within 12 hours show no change in prothrombin time following a small change   With minimal doses, there is a definite decrease of prothrombin time at 48 to 72 hours (figure 1)

Rats fed 2 isovaleryl 1,3 indandione in the diet lived for 5 to 20 days (table 2)   The animals continued to gain weight and showed no symptoms until just before death   Post mortem examination revealed marked hemorrhages, usually in the pleural cavity and lungs but also observed retroperitoneally, subcutaneously, in the cecum thymus eye, etc   The liver was grossly normal but microscopic examination revealed marked centrolobular fatty degeneration and areas of focal necrosis   The blood was incoagulable even after addition of calcium and thromboplastin   Addition of a small amount of fresh serum or of fibrinogen free normal plasma rapidly resulted in coagulation   Addition of fibrinogen was without effect   There was no evidence of the presence of excessive amounts of antiprothrombin or antithrombin in the plasma   These results indicate that the incoagulability of the blood is to be attributed to a disappearance of prothrombin from the plasma   The cumulative effects of repeated subtoxic doses in rabbits is shown in table 3   The terminal reduction in hemoglobin and red cell count is due to hemorrhages occurring just before death

Vitamin K given orally even in very large doses in the form of 2 methyl 1,4 naphthoquinone failed to prevent the rise of prothrombin time and had only a slight effect in prolonging survival of rats receiving as little as 0 005% of 2-isovaleryl 1 3 indandione in the diet (table 2)   In rabbits, a phosphoric ester of

TABLE 1

Effect of oral administration of indandione compounds on prothrombin time in rabbits

COMPOUND	DOSE	PROTHROMBIN TIME (SECONDS)	
		Before administration	Maximum
	mg./kg.		
2-isovaleryl-1,3-indandione	5.0	10.7	10.7
	5.0	10.9	13.8
	7.5	11.4	14.8
	10.0	10.5	15.2
	10.0	10.4	15.9
	15.0	12.7	43.9
	20.0	10.0	26.5
	25.0	12.7	100.0
2-pivalyl-1,3-indandione	1.0	11.3	16.7
	2.0	11.2	13.7
	2.5	10.5	22.9
	5.0	11.5	21.8
	10.0	12.5	18.9
	15.0	11.6	19.5
	25.0	13.1	no clot
2-propionyl-1,3-indandione	10.0	12.7	12.0
	10.0	10.9	18.3
	15.0	14.6	22.3
	20.0	12.5	13.8
	25.0	11.0	19.9
	100.0	12.0	34.5
2-αNaphthoyl-1,3-indandione	1.0	17.4	18.2
	2.0	18.1	25.4
	5.0	16.5	22.4
	10.0	15.9	32.9
	15.0	17.9	93.2
	20.0	18.5	32.2
	25.0	18.4	104.7
1,3-indandione	50.0	12.6	12.6
	50.0	11.8	12.3
	100.0	11.7	10.8
	100.0	15.6	13.4
	500.0	11.4	15.5
2-sodium-2-carbethoxy-1,3-indandione	50.0	11.0	10.9
	100.0	11.6	12.0
	300.0	13.5	12.4
	500.0	11.6	12.8
	1000.0	16.6	54.8
Dibenzoylmethane*	100.0	12.4	11.8
	500.0	11.6	11.8
	1000.0	16.5	24.3

* Not an indandione compound.

K[1] given intravenously in a dose of 35 mg. per kg. failed to prevent a
...othrombin time from a single dose of 10 mg. per kg. of 2-isovaleryl-1,3
ne given orally.   Vitamin K in large doses had a slight effect in decreas-
...ise of prothrombin time resulting from administration of small doses of
...rrhagic agent.

...ute toxicity of large doses of 2-isovaleryl-1,3-indandione was studied in
.. rabbits (table 4).   The animals showed labored respiration, progressive
...weakness, hyperexcitability, pulmonary congestion, venous engorge-
... systolic standstill.   Death occurred in 2 to 12 hours.   As a rule, no
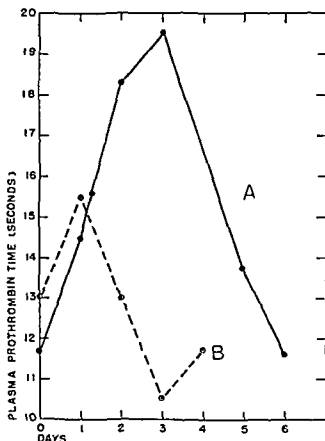
EFFECTS OF INTRAMUSCULAR INJECTION OF 2-ISOVALERYL-1,3-INDANDIONE
IN OLIVE OIL ON PLASMA PROTHROMBIN TIME IN RABBITS
A, 20 mg. per kg.; B, 10 mg. per kg.

... were found.   Following oral administration of 150 mg. per kg. to
...hrombin time was found to be normal at 2.5 to 4 hours but showed a
... ase at 5.5 hours.   Petechial hemorrhages were observed in the lungs
... surviving 12 hours.

...l-1,3-indandione is very slightly soluble in water.   A saturated
...aining 20 mg. per 100 cc. has a distinctly yellow color.   In a cat
... anesthesia a saturated solution of this compound in normal saline
...red intravenously in the course of 2.5 hours.   The total dose given

... 2-methyl-1-4 naphthoquinone diphosphoric ester, Hoffmann-La

was 20 mg. per kg. No significant effects on blood pressure, respiration, or plasma prothrombin time were observed.

TABLE 2

Chronic toxicity of 2-isovaleryl-1,3-indandione when fed in the diet* of rats

NUMBER	WEIGHT	% 2-ISOVALERYL-1,3-INDANDIONE IN DIET	% K† IN DIET	% MORTALITY AT			
				5 to 7 days	8 to 11 days	12 to 15 days	16 to 20 days
	grams						
10	90–100	0.1	0	100			
10	80–100	0.06	0	90	10		
5	50–60	0.01	0	100			
6	120–150	0.01	0	33	33	33	
6	70–85	0.01	0.5	33	33	33‡	
12	80–90	0.005	0	25	33	17	25
12	60–80	0.005	0.2	8	17	42	33§
6¶	80–100	0.1	0	100			
6¶	85–100	0.05	0	100			

* Casein 18, yeast 5, McCollum's salt mixture 4, cod liver oil 2, olive oil 8, starch 63.
† 2-methyl-1,4-naphthoquinone.
‡ Prothrombin time 6¼′, 11′, 3½′, >20′.
§ Clotting time >15′.
¶ 2-pivalyl-1,3-indandione.

TABLE 3

Chronic toxicity of 2-isovaleryl-1,3-indandione in rabbits. Daily oral administrations of 50 mg. per kg. 5% in olive oil

NUM-BER	WEIGHT	HB.	NUMBER OF DOSES	TOTAL GRAMS PER KG.	HEMATOLOGY			SYMPTOMS	SURVIVED OR DIED	NECROPSY FINDINGS
					Hb	R.B.C.	W.B.C.			
	kg.	*grams*			*grams*	*millions per cu. mm.*	*thousands per cu. mm.*			
9	2.5	12.6	4	0.20				None	Died	Thoraci hemorrhage
10	2.4	11.8	8	0.40	10.7	4.9	9.0	Oral hemorrhage	Died	Pulmonary hemorrhage
11	2.5	13.6	8	0.40				None	Died	Pleural effusion & thoracic hemorrhage
12	2.1	12.0	9	0.45	10.5	5.0	21.0	Hemorrhage following venipuncture	Died	Albuminuria
13	2.4	14.9	6	0.30	9.3			Nasal and oral hemorrhages	Died	Pulmonary hemorrhages

The effect of addition of 2-isovaleryl-1,3-indandione to normal plasma *in vitro* was also investigated. There was no immediate effect on prothrombin

time when rabbit plasma was saturated with this compound, while on standing, there was a rapid deterioration of prothrombin and consequent elevation of prothrombin time even at 30°C (fig 2)   Normal rabbit plasma shows no change in prothrombin time in two hours at room temperature (12)   Human plasma saturated with 2 isovaleryl 1,3 indandione shows complete disappearance of

TABLE 4

Acute toxicity of indandione derivatives administered in olive oil orally

RATS		DOSE	% MORTALITY
Number	Weight		
A 2 isovaleryl 1 3 indandione			
		mg/kg	
5	200–230 gm	100	20
8	220–260 gm	200	100
6	210–285 gm	300	100
RABBITS			
4	2 0–2 5 kg	100	0
3	1 2 1 5 kg	150	67
3	1 7–1 8 kg	200	100
B 2 pivalyl 1 3 indandione			
RATS			
4	150–200 gm	100	0
5	140–200 gm	150	80
3	200–250 gm	200	100
3	215–235 gm	400	100
RABBITS			
3	1 5–3 0 kg	100	33
3	1 4–2 2 kg	150	33
3	1 3–1 5 kg	200	100
C 2 propionyl 1 3 indandione			
RATS			
10	110–160 gm	300	0
5	105–164 gm	400	80
8	65 176 gm	500	75
9	70–156 gm	1000	100

prothrombin after standing for two hours, the plasma clots promptly when fresh serum is added   Saturated 2 isovaleryl 1,3 indandione plasma diluted 1 10 with normal human plasma also shows more rapid deterioration of pro thrombin *in vitro* than normal (fig 3)   On the other hand, saturated 2 iso valeryl 1,3 indandione plasma diluted 1 50 with plasma shows less rapid deterior
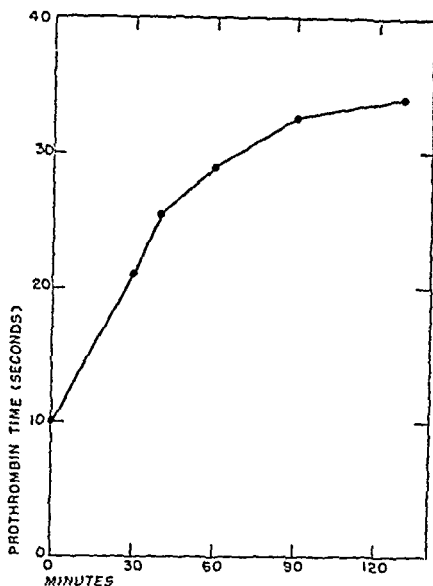
FIG. 2. THE EFFECT OF STANDING AT 30°C. ON PROTHROMBIN TIME AFTER SATURATION OF NORMAL RABBIT PLASMA WITH 2-ISOVALERYL-1,3-INDANDIONE IN VITRO
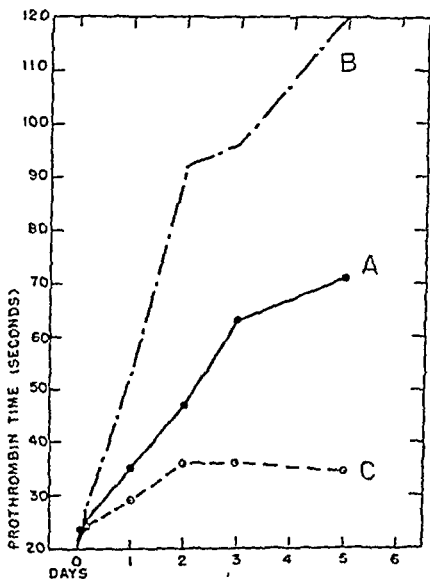
FIG. 3. EFFECT OF STANDING ON PROTHROMBIN TIME OF NORMAL HUMAN PLASMA AFTER ADDITION OF 2-ISOVALERYL-1,3-INDANDIONE IN VITRO

A, normal plasma; B, plasma saturated with 2-isovaleryl-1,3-indandione diluted 1:10 with normal plasma; C, plasma saturated with 2-isovaleryl-1,3-indandione diluted 1:50 with normal plasma.

ation of prothrombin than normal (fig. 3). It would seem that the stability of plasma prothrombin is increased by low concentrations of 2-isovaleryl-1,3-indandione, while higher concentrations have the opposite effect.

(B) 2-pivalyl-1,3-indandione

$$CH \cdot CO \cdot C(CH_3)_3$$
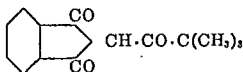
This compound has a more pronounced effect in depressing plasma prothrombin than 2-isovaleryl-1,3-indandione. The minimal effective oral dose in rabbits is less than 1 mg. per kg. which is in the same range of effectiveness as dicumarol (1). Even as little as 0.25 mg. per kg. increases prothrombin time of diluted plasma. A dose of only 25 mg. per kg. orally renders the blood prothrombin-free in 72 hours (table 1). The effects of incorporating 2-pivalyl-1,3-indandione in the diet of rats was in every way similar to that of 2-isovaleryl-1,3-indandione (table 2). The acute toxicity of 2-pivalyl-1,3-indandione is also similiar to that of 2-isovaleryl-1,3-indandione except that petechial hemorrhages were observed in the lungs of rats which died 1 to 2 hours after administration of a single large dose.

(C) 2-propionyl-1,3-indandione

$$CH \cdot CO \cdot C_2H_5$$
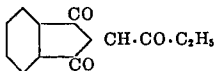
This compound is similar to the isovaleryl and pivalyl indandiones but less active. The effects of various doses on prothrombin time in rabbits is shown in table 1. The minimal effective oral dose is 10 to 15 mg. per kg. The prothrombin time is increased to a greater extent by 25 mg. per kg. of 2-isovaleryl-1, 3-indandione or 2-pivalyl-1,3-indandione than by 100 mg. per kg. of 2-propionyl-1,3-indandione. The acute toxicity data are incorporated in table 4.

(D) 2-Naphthoyl-1,3-indandione

$$CH \cdot CO \cdot$$
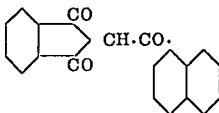
This compound is highly active in depressing the plasma prothrombin level (table 1). The minimal effective oral dose is 2 mg. per kg.

(E) 1,3-indandione

$$CH_2$$
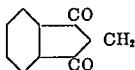
As can be seen from table 1, this compound is relatively ineffective in prolonging prothrombin time. The minimal effective dose in our series was 500 mg. per kg.

(F) 2-Na-2-carbethoxy-1,3-indandione
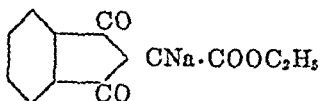
This compound is water soluble. It is relatively ineffective in elevating prothrombin time (table 1). No significant change in prothrombin time is observed until 1 gram per kg. is administered.

(G) Dibenzoylmethane
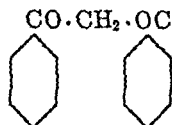
This compound was used to determine the effectiveness in reducing plasma prothrombin of a substance containing the methylene bridge of dicumarol and two ketone groups. This compound is relatively ineffective (table 1), the minimal dose to prolong prothrombin time being 1 gram per kg.

DISCUSSION. The series of compounds studied in this paper for their activity in inducing hypoprothrombinemia is of considerable interest for the elucidation of the problem of the relation of chemical structure to this specific activity. The compounds may be divided into two groups. One group, which is highly active in inducing hypoprothrombinemia, consists of 2-isovaleryl-1,3-indandione, 2-pivalyl-1,3-indandione, 2-propionyl-1,3-indandione and 2-anaphthoyl-1,3-indandione. While the character of the side chain results in some variation in activity, all of these compounds produce a marked hypoprothrombinemia with relatively small doses. One of these compounds, 2-pivalyl-1,3-indandione is as effective a hemorrhagic agent as dicumarol. The second group of compounds has extremely weak hypoprothrombinemic activity and consists of 1,3-indandione, 2 sodium-2-carbethoxy-1,3-indandione and dibenzoylmethane. These compounds differ from those of the first group in containing two instead of three ketone groups. The third ketone group therefore assumes considerable importance in determining the effect on plasma prothrombin.

In the series of coumarin compounds studied by Lehmann (6), only the dicoumarins with two ketone groups and two enol groups were highly active, while the coumarins with one ketone and one enol group required very large doses to produce a reduction in plasma prothrombin. Link and his co-workers (7) made the observation that salicylates produce hypoprothrombinemia when relatively large doses are administered. They state that only those compounds show anticoagulant action which theoretically might yield salicylic acid on

degradation   The indandione compounds are more likely to yield phthalic acid as a degradation product rather than salicylic acid   Moreover, assuming the theoretical possibility of salicylic acid as a degradation product of the active indandiones, there is no reason to suppose that the inactive ones would not undergo similar degradation   Furthermore, a small amount of vitamin K will prevent the hypoprothrombinemia from a large dose of salicylates, while vitamin K is quite ineffective in antagonizing dicumarol and the indandiones   It there fore appears unlikely that the active indandione compounds and dicumarol exert their effect in prolonging prothrombin time by being metabolized in the body to salicylic acid   Dibenzoylmethane exerts a slight hypoprothrombinemic effect despite the fact that it probably could not be degraded to salicylic acid

The observation that 2 isovaleryl-1,3 indandione added to plasma *in vitro* has a significant effect in accelerating the deterioration of prothrombin on standing may be of importance in elucidating the mechanism of action of the indandione compounds and dicumarol   Campbell and his co workers (13) observed that prothrombin in plasma from rabbits fed spoiled sweet clover hay (containing dicumarol) was less stable than normal plasma   A rapid decrease in prothrombin activity of pathological plasma incubated at 37°C occurred in 4 to 8 hours, while little or no change in the prothrombin time of normal plasma was noted These observations on 2 isovaleryl-1,3-indandione and dicumarol indicate that the stability of prothrombin in plasma may be affected by these hypoprothrombinemic agents as well as the possible effect on liver production of prothrombin

SUMMARY AND CONCLUSIONS

1   The effectiveness of a number of indandione derivatives in inducing hypoprothrombinemia has been investigated   The action of these compounds is similar to that of dicumarol   One of these compounds appears to be as potent a hemorrhagic agent as dicumarol

2   Compounds with three ketone groups were found to be highly active   On the other hand, compounds with two ketone groups showed only slight activity

3   Addition of 2 isovaleryl-1,3 indandione to plasma *in vitro* results in instability of the plasma prothrombin

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A CONTRIBUTION TO THE PHARMACOLOGY OF THE NITRATE ESTER OF CHOLINE PERCHLORATE[1]

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Although the esters of choline containing organic acids have been investigated extensively in many laboratories, the inorganic choline esters have not enjoyed the same consideration Nitrosocholine, the nitrous acid ester of choline, called at one time pseudomuscarine, however, has been studied by Ewins (1) He found it to be about 500 times more potent than choline, it possessed curare-like activity and produced no miosis The authors have been concerned with the pharmacology of *Xanthium spinosum* (2) and from the burs of the plant have succeeded in isolating choline In the identification of the base, the nitrate ester of choline perchlorate was prepared according to the method of Hofman and Hobold (3) The paucity of information on the inorganic esters of choline and the stability of the salt prompted the investigation of this ester

Physical and chemical characteristics The nitrate ester of choline perchlorate is a white crystalline compound which melts between 188 and 189°C The crystals are highly double refractive, stable in air and easily purified by recrystallization from hot water A saturated aqueous solution at 20°C contains 0 82 gram per 100 cc The structure of the ester is

$$CH_3-N-CH_2\cdot CH_2\cdot ONO_2$$

with CH_3, CH_3, and ClO_4 attached to the nitrogen.
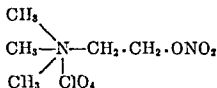
Microanalyses in duplicate yielded the following results as compared with the values calculated for the foregoing formula

Calculated C, 24 15 per cent, H, 5 27 per cent, N, 11 27 per cent

Found C, 24 95 per cent, 24 93 per cent, H, 5 61 per cent, 5 50 per cent, N, 11 06 per cent, 11 20 per cent

Aqueous solutions of the ester appear to retain their pharmacologic activity over long time periods The pH of a solution, 1 1000, lies between 6 and 7

Miosis in the rabbit Twelve rabbits received 2 drops of 1 per cent solution of the nitrate ester of choline perchlorate instilled into the conjunctival sac of one eye The contra lateral eye served as a control The treated eyes in all cases exhibited a strong pupillary constriction within 10 minutes which was antagonized by atropine Four additional rabbits received acetylcholine in

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the same manner. No miosis was elicited. Molitor (4) observed that miosis was not produced until the concentration of acetylcholine was 5 per cent.

Depressor response. In dogs and cats (10 experiments) the depressor response upon intravenous injection of the nitrate ester was approximately 50 per cent
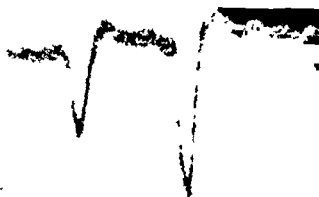
CHART 1. BLOOD PRESSURE, DOG, 6 KG. ETHER ANESTHESIA
No. 1. 1 cc. I.V. nitrate ester of choline perchlorate 1 in 10⁵.
No. 2. 1 cc. I.V. acetylcholine 1 in 10⁵.

that of acetylcholine when compared in concentration of 1 in 10^5. The depressor response was obliterated by atropinization and there was some evidence of nicotine-like action in certain of the animals after atropinization.

The relative response of the esters on the blood pressure of the dog, ether anesthesia, is shown in chart 1.

The depressor activity of the nitrate ester of choline perchlorate is not diminished by incubation for 1 hour with the dog's blood serum, indicating its refractoriness to the choline esterase.
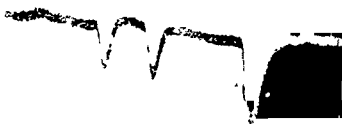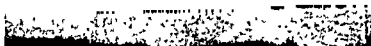
CHART 2. EFFECT OF CHOLINE ESTERASE. DOG, 5 KG., ETHER ANESTHESIA
No. 1. with serum.
No 2 after 1 hour at 37°C.
No. 3
No. 4.

This effect compared with acetylcholine is shown in chart 2.

Action on smooth muscle. The action of the nitrate ester of choline perchlorate on the pylorus of the frog was studied *in vitro*. The relative effects of the ester and acetylcholine are shown in chart 3.

LD-50 nitrate ester of choline perchlorate.   The LD-50 (one hour) of the nitrate ester of choline perchlorate was determined on the rat by intraperitoneal injection and compared with that of acetylcholine.   The results are shown in table 1.

It is apparent from the data in table 1 that the LD-50 (one hour) for acetylcholine for the rat upon intraperitoneal injection is approximately 25 mg./100 grams
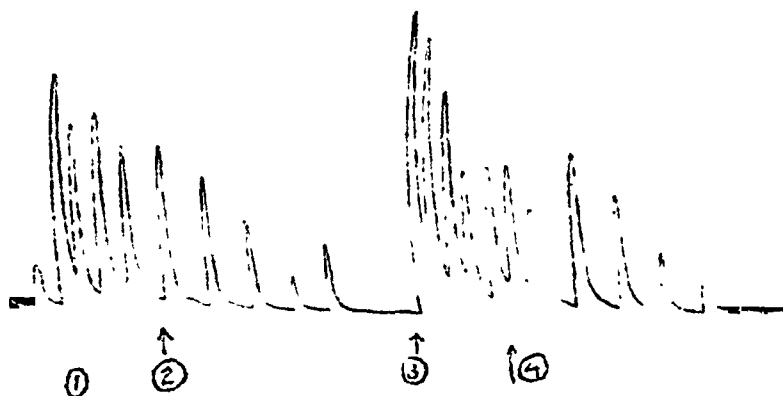
CHART 3. EFFECT OF CHOLINE ESTERS ON FROG'S PYLORUS
1. Acetylcholine, 1 in 2.5×10^5.   2. Tyrode's soln.   3. Nitrate ester of choline perchlorate, 1 in 2.5×10^5.   4. Tyrode's soln.

TABLE 1

Comparative intraperitoneal toxicity of nitrate ester of choline perchlorate and acetylcholine for rats

ACETYLCHOLINE			NITRATE ESTER OF CHOLINE PERCHLORATE		
Mg /100 gm.	Survived	Died	Mg /100 gm.	Survived	Died
30	0	5	40	0	1
25	6	4	30	0	1
20	9	1	20	0	1
15	5	0	15	1	3
10	5	0	10	0	5
			5	1	4
			2.5	4	6
			1	4	1

body weight.   The value assigned to acetylcholine for mice by Morrison (5) is 20 mg./100 grams of body weight.   The approximate comparative LD-50 of the nitrate ester of choline perchlorate is 2.5 mg./100 grams of body weight.

The deaths produced by the nitrate ester were much more prompt than those caused by acetylcholine.   The convulsive properties of acetylcholine are far less pronounced than those of the nitrate ester.   The latter produces convulsions

within 3 minutes when administered in lethal doses   The convulsions were clonic and characterized by marked emprosthotonus   They lasted no longer than 1 minute and invariably at their termination the animal died

Chromodacryorrhea test   The nitrate ester of choline perchlorate, in doses from 1 to 40 mg per 100 grams, produced "bloody tears" in 15 rats within one minute Acetylcholine injected intraperitoneally in quantities of 10 to 30 mg per 100 grams gave immediate evidence of chromodacryorrhea   The chromodacryorrhea produced by the nitrate ester was obliterated by intraperitoneal injections of atropine sulfate (10 mg per 100 grams)

SUMMARY

1 The nitrate ester of choline perchlorate is a stable inorganic acid ester which mimics many of the pharmacological responses of acetylcholine

2 Its depressor potency is approximately one-half that of acetylcholine   Its depressor effect is obliterated by atropine but not affected by the choline esterase

3 This ester is approximately ten times as toxic to the rat as is acetylcholine

4 It is possible that this simple choline ester may find application in therapeutics as a parasympathomimetic drug

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STUDIES ON THE ACTION OF MORPHINE ON THE CENTRAL NERVOUS SYSTEM OF CAT

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The loci and mechanism of action of morphine on the central nervous system and the reasons for species differences are still incompletely understood. As Cushny (1) states, "It is impossible to state any general theory of the action of morphine on nerve cells which covers these differences in the behavior of different animals and also in the reactions of different nerve centers in the same animal." The generalization that "morphine depresses from above downward and stimulates from below upward" (2) is inadequate since both "depressant" and "stimulant" effects can be demonstrated at all levels in the nervous system. Inferences regarding the central nervous system action of the drug which are based on observations of the effects on intact animals are hazardous since the animal reacts as a total organism and it is therefore difficult to distinguish between direct stimulant actions, "release" phenomena secondary to selective depressions and compensatory reactions. Studies on the effects of morphine after ablation of various parts of the central nervous system yield more information concerning its action on specific neurones, although the effects observed may not be significant for the intact animal because of the influences, facilitating or inhibiting, of the parts removed.

The literature has been exhaustively summarized by Eddy (3). Of particular relevance to this report are the investigations of Joel and Arndts (4) and Tatum, Seevers and Collins (5). These authors have emphasized the biphasic actions of moderate doses of morphine, namely initial depression followed by excitation, the relative intensity of the two varying with the species. With small doses the *depressant effect may be long and the subsequent excitant action hardly de*tectable, but with large doses the initial depression may be very short, convulsions appearing soon after injection. However, as Tatum et al, indicate, the initial action of small or moderate doses is not one of pure depression but the activity of some portions of the nervous system is depressed while that of other parts is enhanced at the same time. No hypothesis has yet been advanced explaining the stimulant and depressant actions in terms of the effects of morphine on different types of nerve cells or groups of cells which would be valid at all levels of integration.

The experiments which form the basis of this report constitute the preliminary portions of a systematic reinvestigation of the effects of morphine on the central nervous system of various laboratory animals and man, and hence will be described in considerable detail. Some of the results on the effects of small and

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moderate doses on acute decorticate, hypothalamic, decerebrate, spinal and chronic spinal cats are in agreement with reports in the literature but others are in apparent conflict Careful consideration of these similarities and differences may help clarify some of the problems concerning the action of single doses of morphine on the nervous system

METHODS Forty-nine cats were studied, distributed as follows intact—9, acute decorticate—12, acute hypothalamic—6, acute decerebrate—6, acute spinal—12, chronic spinal—4 In addition, many of the acute spinal preparations were made by preliminary decerebration followed by transection of the cord in the mid-thoracic region Such preparations were decerebrate above the level of transection and spinal below Details of the methods used are as follows

Intact cats were observed before and after the injection of morphine either alone or in the company of other normal or morphinized cats under various conditions such as confinement in small laboratory cages, glass walled boxes or large observation cages made of wire netting The responses of the animal to handling noise, the presence of mice and other stimuli were noted

Decorticate preparations were made essentially as described by Bard (6) Under light ether anesthesia the carotid arteries were ligated and the trachea cannulated The scalp and temporal muscles were incised and reflected The calvarium was then trephined and removed The dura was incised and both hemispheres, except for the pyriform lobes and possibly parts of the gyri prorei, were removed by dissection with blunt spatulas, leaving the corpus callosum and subjacent structures intact Bleeding was controlled during the operation by pressure applied intermittently to the vertebral arteries just above the transverse processes of the atlas After decortication vertebral artery pressure was discontinued entirely and bleeding controlled by cotton packs The animal was not restrained, but the head was supported by a string passed through the cut edges of the scalp

Acute hypothalamic preparations were made as above, but after decortication, another transection was made along an oblique plane from the dorsal aspect of the superior colliculi to the optic chiasm, thus removing the basal ganglia, most of the thalamus and the hippocampus on both sides

Acute decerebrate animals were made by exposing the cortex as already described The occipital lobes were then retracted, the falx incised and the brain transected just anterior to the vertical plane between the superior and inferior colliculi, the contents of the skull rostral to this plane were removed Observations were carried out in a warm room and external heat was applied to the animal during the experiment

Acute spinal preparations were made by preliminary decerebration, followed by transection of the spinal cord either at the level of the atlas or the seventh thoracic vertebra Artificial respiration was maintained in the former Chronic spinal preparations were made by aseptic transection of the spinal cord at the level of the seventh thoracic vertebra under sodium amytal or pentobarbital anesthesia, the preliminary laminectomy being limited to one or two vertebrae In some experiments acute spinal animals were prepared for iso metric kymographic recording of the knee jerk, and the flexor (tibialis anticus) reflex, after denervation of all other muscles of the limb The knee jerk was elicited by means of a pendulum hammer which could be made to fall from a fixed level at any desired interval The tibialis anticus flexor response was obtained by stimulation of the homolateral plantar or internal saphenous nerves by single break shocks obtained from a coreless induction coil

The dose of morphine used in most of the experiments was 5 mgm per kg Observations were also made on the effects of doses ranging from 2 15 mgm per kg The route of administration was usually intravenous, a 1% solution being used and injected slowly over a period of two to five minutes Experiments were also made with subcutaneous injections in preparation of each type In several experiments the femoral or carotid arteries were cannulated for kymographic recording of the blood pressure

STUDIES ON THE ACTION OF MORPHINE ON THE CENTRAL NERVOUS SYSTEM OF CAT

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The loci and mechanism of action of morphine on the central nervous system and the reasons for species differences are still incompletely understood. As Cushny (1) states, "It is impossible to state any general theory of the action of morphine on nerve cells which covers these differences in the behavior of different animals and also in the reactions of different nerve centers in the same animal." The generalization that "morphine depresses from above downward and stimulates from below upward" (2) is inadequate since both "depressant" and "stimulant" effects can be demonstrated at all levels in the nervous system. Inferences regarding the central nervous system action of the drug which are based on observations of the effects on intact animals are hazardous since the animal reacts as a total organism and it is therefore difficult to distinguish between direct stimulant actions, "release" phenomena secondary to selective depressions and compensatory reactions. Studies on the effects of morphine after ablation of various parts of the central nervous system yield more information concerning its action on specific neurones, although the effects observed may not be significant for the intact animal because of the influences, facilitating or inhibiting, of the parts removed.

The literature has been exhaustively summarized by Eddy (3). Of particular relevance to this report are the investigations of Joel and Arndts (4) and Tatum, Seevers and Collins (5). These authors have emphasized the biphasic actions of moderate doses of morphine, namely initial depression followed by excitation, the relative intensity of the two varying with the species. With small doses the depressant effect may be long and the subsequent excitant action hardly detectable, but with large doses the initial depression may be very short, convulsions appearing soon after injection. However, as Tatum et al, indicate, the initial action of small or moderate doses is not one of pure depression but the activity of some portions of the nervous system is depressed while that of other parts is enhanced at the same time. No hypothesis has yet been advanced explaining the stimulant and depressant actions in terms of the effects of morphine on different types of nerve cells or groups of cells which would be valid at all levels of integration.

The experiments which form the basis of this report constitute the preliminary portions of a systematic reinvestigation of the effects of morphine on the central nervous system of various laboratory animals and man, and hence will be described in considerable detail. Some of the results on the effects of small and

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and could not be elicited by any stimuli   Bradycardia and slowing of respiration appeared during or occasionally a minute or two after intravenous injection No cyanosis or pallor of the buccal mucosa or tongue were observed   Except for small, transitory fluctuations during the first few minutes following intravenous injection, the blood pressure was not altered significantly   The pupils became dilated a few minutes after injection and this was reduced only slightly by section of the cervical sympathetic trunk   Righting reflexes and running movements were abolished   A striking effect in most experiments was the early appearance of marked extensor rigidity of the forelegs   Frequently the tonic neck and labyrinthine reflexes of Magnus and De Kleijn could be demonstrated, the whole picture resembling strongly that of a decerebrate preparation   In some experiments running movements returned approximately one hour after intravenous injection but in most cases recovery did not begin until several hours had elapsed and "sham rage" did not reappear until four or five hours after injection   In a few experiments with larger doses recovery from the depressive effects occurred more rapidly (within an hour) and subsequently running movements appeared to be enhanced   Injection of a second dose of morphine after recovery from the first would occasionally fail to reproduce the depressive effects of the first dose Intravenous injections of subconvulsive doses of metrazol or ephedrine restored the picture of " sham rage" to a large extent when administered during the period of morphine depression

   C Acute hypothalamic preparations   After recovery from ether the animal usually exhibited the classic picture of ' sham rage', running movements, and righting reflexes which could not be distinguished by gross inspection from those shown by the decorticate preparation except that the pupils were often unequal and unresponsive to light due, probably, to injury to the protectal region or the nerves around the superior orbital fissure

   Injection of morphine was followed by the same effects as in the decorticate preparations except that the abolition of ' sham rage" and running movements was often incomplete and recovery was much more rapid, occasionally beginning as soon as twenty minutes after completion of the injection   The effects of second injections and of metrazol or ephedrine were similar to those seen in the decorticate animals

   D Acute decerebrate preparations   After recovery from anesthesia, marked extensor rigidity of all four limbs and the tail appeared regularly   The tonic and labyrinthine reflexes were readily obtained   The pupils were usually in mid dilatation but unresponsive to light   Responses to noxious stimuli were inconstant and variable   Frequently there was definite diminution in extensor tone of the homolateral limb on pinching or clamping the footpads, but on other occasions no effect was observed   The animal could stand but not walk   In a few preparations even before injections of morphine rhythmic alternating movements of the extended forelimbs pivoting at the shoulder, were seen   These were inhibited by pinching the footpads and this effect was accompanied by slowing of respiration and even apnea during the application of the noxious stimulus

Following morphine, the extensor tone of the limbs was enhanced in some animals but unaffected in others. Occasionally during intravenous injection a tonic extensor seizure was observed. One preparation died immediately after such an episode; in those that exhibited the phenomena and survived, no essential difference from the pre-injection status was seen. The labyrinthine reflexes were unaltered by morphine while the tonic neck reflexes were less easy to demonstrate in some experiments, but unchanged in others. After the lapse of approximately one-half to one hour after morphine, rhythmic alternating movements of the forelegs similar to those described above were seen regularly. These too, could be inhibited easily by exteroceptive stimuli applied to the forelegs, such as touching, pinching or pressure on the footpads. On the other hand, the frequency of these movements was greatly increased by removing such stimuli as for example by holding the animal in the air suspended by the head and tail. Concomitantly these preparations also exhibited a synchronous twitch of the extremities when the table on which they rested was struck suddenly. The response was similar to the "startle response" of the intact animal. Reflex turning of the head in response to loud and sudden noises or blowing on the face or ears was enhanced. The alternating movements of the forelegs, "startle" "response" and reflex turning of the head in response to noise and blowing on the face, while delayed in onset increased with time. Convulsions, however, did not occur with the dose used.

E. Acute and chronic spinal preparations. In the acute preparation brisk knee and ankle jerks as well as active flexor and crossed extensor reflexes appeared within an hour after operation. Transection in the midthoracic region was observed to have an immediate augmenting effect on the decerebrate extensor rigidity of the forelegs. In the chronic preparations the stretch reflexes (knee and ankle jerks) and reflexes to nociceptive stimuli (flexor and crossed extensor) were active after recovery from barbiturate anesthesia.

Within a minute or two following injection of morphine the flexor and crossed extensor reflexes disappeared, while the knee jerks remained unchanged or were slightly enhanced. Records of the knee jerk showed a striking uniformity in size of the response after morphine. The tibialis anticus response to single faradic break shocks applied to the homolateral plantar or internal saphenous nerves were markedly reduced but not abolished. The selective depression of the responses to noxious stimuli was seen in all spinal preparations, acute and chronic. In the chronic preparations the usual effects of morphine on the intact animal were readily apparent above the level of transection of the spinal cord concomitant with the changes observed below that level, indicating that circulatory or anoxic changes were not responsible for the changes in spinal reflexes. In the acute preparations kymographic records of the blood pressure showed no significant change after intravenous injection of morphine. As in other preparations the rate of recovery was variable, usually from one to four hours. The effects of second injections of morphine after recovery from the first injection, and the effects of injections of metrazol or ephedrine during the period of morphine depression of the flexor reflex were comparable to those observed at other levels.

DISCUSSION The intravenous route of administration of a drug like morphine is of advantage in studying effects on acute preparations since the "base line" of such animals may change significantly enough within an hour to complicate the results. On the other hand, the possibility of blood pressure changes in affecting the results must be considered since Schmidt and Livingston (7) have reported that first intravenous injections of morphine causes a marked fall in blood pressure in cats and dogs due to a peripheral vasodilator action. However under the conditions of our experiments such marked changes in blood pressure were not observed with the doses used. In decorticate cats, following intravenous injection of 5 mgm per kg, an initial drop of twenty to thirty mm Hg was observed but the blood pressure rapidly returned to normal and remained steady at or slightly above the pre-injection level 2½ minutes after completion of the injection. Furthermore the effects on the central nervous system were qualitatively the same with doses of two mgm per kg, which from the data of Foster (3) could be expected to cause only negligible changes in blood pressure. In our spinal cats intravenous injection of 5 mgm per kg of morphine caused no change in blood pressure at all. The pre-injection blood pressure level in the acute spinal preparations was already low (usually sixty to seventy-five mm Hg) and it appears probable that peripheral vascular tone is naturally at a minimum in these preparations. In one case the blood pressure remained at a steady level of 40 mm Hg, yet the flexor reflex and knee jerk were brisk before injection of morphine. Furthermore control experiments on decorticate and spinal cats with subcutaneous injections yielded the same results as were obtained with intravenous injections, except that in the former the effects on the central nervous system began to appear 15 or 20 minutes after injection while in the latter the changes began almost immediately. Conversely, the effects were more prolonged after subcutaneous injection than after intravenous injection. These observations are in accord with the findings of Seevers and Pfeiffer (8).

The effects of morphine on the intact cat are usually interpreted in terms of "stimulation" and are attributed to some peculiarity in the reaction of the nerve cells of this species to the drug, although "depressive" effects, such as failure to avoid obstacles have been noted (1). On the other hand, Straub (9) regarded the "excitant" action on the intact cat as due to a weak cortical depressant action, "releasing" primitive patterns of behavior. Joel and Arndts (4) on the other hand held that the sensitivity of the cat to morphine approached that of man and that the "stimulant" effects (of 10 to 20 mgm per kg) were due to the fact that such doses were far too high for this species. These authors claimed to have observed distinct narcotic effects with very small doses (0 1–0 2 mgm per kg). This has been denied by Eddy (3). Our observations are in general agreement with those of Joel and Arndts. On the other hand some of the "excited" behavior of the cat after injection of even small doses (5 mgm per kg) such as the mad scrambling to escape when handled or confined in a small cage, fear of the experimenter and darting under radiators and other objects when released, appear to represent disintegration of adaptive behavior (10), rather than "stimulation." Such behavior could be due to depressive effects exerted selectively on the integrative functions of the cortex as well as

elsewhere in the nervous system.   An analogy may be drawn between the excitant effects of small doses of alcohol on some human individuals and the action of moderate doses of morphine on the intact cat.   However the exaggerated motor restlessness and muscle twitches appearing in the latter may be due to true delayed stimulant actions of the drug.

The effects of small doses of morphine on the decorticate cat are again predominantly depressant, while the concomitant "stimulant" effects such as extensor rigidity of the forelegs are strikingly like those seen after decerebration in normal cats and can therefore be interpreted as "release" phenomena.   Delayed stimulant actions such as were seen by Joel and Arndts after injection of 2 mgm. per kg. subcutaneously, were not seen in our experiments even after 5 mgm. per kg.   On the other hand after 15 mgm. per kg. (subcutaneous) delayed running movements appeared which were more active than before injection, but which were inhibited rather than augmented by noxious stimuli.   It appears remarkable that Joel and Arndts did not describe "sham rage" or the effects of morphine thereon in their acute "thalamic" preparations.   In view of this fact, the nature of the stimulant effects observed by them as late as 10 hours after injection is open to question, since their appearance may have been due to delayed recovery from the operation.   Our observations are otherwise in accord with the effects they described as characteristics of the "first" or depressant stage.

The depressant effects seen in our experiments seem to conflict with the reports of excitant actions of morphine on chronic decorticate cats by Hambourger (11) and Brooks, Goodwin and Willard (12).   This disagreement is probably more apparent than real and may be due to (a) differences in dosage, (b) time of onset of observed effects and (c) differences in "base line", i.e. our acutely decorticated animals were very active, exhibiting "sham rage" spontaneously and in response to painful stimuli before injection of morphine, whereas the chronic decorticated cats (Hambourger) were quiet.   The excitant effects reported in the latter consisted in increased muscle tone and running movements, not "sham rage".   The importance of dosage is shown by Girndt's (13) observation that small doses of morphine (0.1 to 0.2 mgm. per Kg.) had depressant effects on chronically decorticated cats while larger doses (above 1 mgm. per Kg.) caused "excitement".   Amsler's (14) report on the ineffectiveness of morphine as an analgetic after chronic decortication (guinea pigs, rats and dogs) may possibly be due to similar factors.   On the other hand, Mettler and Culler (15) found that the narcotic effects of morphine on the dog were greatly enhanced after decortication.

The effects observed in the hypothalamic preparations are essentially the same as in the decorticate animals except that the duration of the depressant actions was usually shorter.   This may be due to further "release" of lower centers by ablation of the thalamus and striatum or to an action of morphine on these structures which was eliminated by their removal.   The first seems to be the more likely explanation since the effects of morphine differed only quantitatively from those observed in the decorticate animal.   It should be emphasized here that only the complex skeletal motor components of the "sham rage" pattern, namely,

clawing, snarling, scratching and springing movements were depressed by morphine, while spontaneous autonomic activity was not so affected Thus, blood pressure appeared to be unchanged but the pupils were dilated and some degree of piloerection was present even though the animal appeared to be narcotized and unresponsive to stimuli after injection of morphine The pulse and respiration were slowed but these were probably due to medullary effects since these actions have been observed in acute decerebrated animals (16, 17) Likewise, Brooks, Goodwin and Willard (12) reported that morphine hyperglycemia is due to a direct stimulant action on autonomic centers in the hypothalamus, since it was abolished after a lesion was made in the posterior hypothalamus On the other hand, the "motor excitement" (running movements ?) produced by morphine was not abolished by such a lesion and Hambourger (18) obtained inconclusive results by similar procedures, while Masserman (19) found that morphine did not alter the excitability of the hypothalamic nuclei to faradic stimulation It appears therefore that the hypothalamic origin of the skeletal motor excitant effects reported by these authors has not been demonstrated conclusively Our observations point to the posterior portion of the midbrain as the site of the delayed excitant effects (running movements) of morphine, in the doses used

The depression of righting reflexes and appearance of extensor rigidity after injection of morphine in the acute decorticate and hypothalamic animal points to the reticular substance of the midbrain as another important locus of action of the drug However, in view of the work of Fulton and Connor (20) on the inhibitory function of the anterior lobe of the cerebellum on decorticate rigidity, a depressant effect of morphine on this organ cannot be excluded Furthermore, the rigidity seen in our experiments after morphine differs from the 'decorticate rigidity" of Bard and Rioch (21) since it was not diminished by pressure on the footpads

The absence of depressive effects on the extensor rigidity of the decerebrate preparation and on the labyrinthine reflexes indicates that the vestibular nuclei are not depressed by morphine The nature of the rhythmic alternating movements of the forelegs in these preparations is not entirely clear They resembled running movements modified by extensor rigidity and may be analogous to the delayed augmented running movements seen after larger doses of morphine in the decorticate and hypothalamic preparations Their greater intensity and earlier onset in the decerebrate preparation may be due to further release effects consequent to intercollicular section On the other hand since Bailey and Davis (22) produced a ' Syndrome of Obstinate Progression" in cats by an electrolytic lesion on the interpeduncular nucleus of the midbrain, the possibility that these running movements are also "release phenomena" is not excluded Whichever explanation is correct, it appears probable that the production of running movements is not due to a specific property of morphine since Brown (23) described "narcosis progression" in decerebrate and spinal animals under chloroform anesthesia and similar movements are often seen during recovery from ether anesthesia in both dogs and cats

The absence of depressant effects of morphine on the knee jerk is in conflict

with the report of Luckhardt and Johnson (24), who found marked depressant effects and with the report of Bodo and Brooks (25) who noted slight depressant effects. The experiments on cats performed by Luckhardt and Johnson were all done under barbiturate anesthesia, which may have influenced their results. On the other hand, differences in dosage may explain some of the discrepancies, since our observations were made chiefly with small and moderate doses. Contrary to the statements in most textbooks there is agreement among most modern investigators that the flexor reflex is depressed by morphine in both cats and dogs. In cats Blume (26) found that small doses depressed the flexor reflex, moderate doses had a biphasic action (depression followed by stimulation) while large doses augmented the flexor reflex and finally caused tetanic convulsions. In a few of our experiments after recovery from the depressant effects of 5 mgm. per kg. of morphine (intravenous) the flexor reflex seemed to be more active than before injection.

The differential effect of morphine on nociceptive and myotatic reflexes observed in our experiments may be explained on the basis that morphine depresses pain fibers in the periphery. This explanation while not entirely invalidated, is not strongly supported by the available evidence in the literature. Two other explanations, while still hypothetical, seem to fit the facts. One, along lines similar to those suggested by Tatum, Seevers and Collins (5) would be that morphine simultaneously stimulates anterior horn cells and depresses internuncial neurones. Such differential effects need not be ascribed to any special "dual" action of morphine, but could be due to inherent differences (e.g. in metabolism or blood supply) in the two types of cells. The other, that the primary action of morphine is that of a mild depressant on internuncial neurones (or on all neurones in general), the "stimulant" effects on anterior horn cells being due to "release" of the latter from the inhibitory activity of some internuncials. The importance of the role of the internuncial neurones in explaining differential actions of drugs on the spinal reflexes tends to be supported by the demonstration by Lloyd (27, 28) that the myotatic reflex traverses a two neurone arc involving no internuncials while the flexor reflex passes over a multineurone arc involving one or more interneurones. The differential effect of eserine on the reflexes of spinal dogs (knee jerk depressed, flexor reflex enhanced) was attributed to the effect of the drug on internuncial neurones by Merlis and Lawson (29). Kabat and Knapp (30) have attributed the muscular spasticity often seen in poliomyelitis to augmented stretch reflexes which they correlated with pathological lesions in the internuncial neurones of the spinal cord.

The generalization that interneurones are depressed while motoneurones are stimulated (directly or by release from inhibitory activity) by small doses of morphine seems also to be compatible with the effects noted at higher levels. Thus righting reflexes, locomotion and the "sham rage" pattern, which are complex responses undoubtedly integrated through many interneurones, are depressed, whereas less complex responses, presumably integrated over fewer neurones, such as the tonic neck and tonic labyrinthine reflexes are unaffected.

Similarly, although there may be depressant effects on complex, highly integrated cortical functions such as learned adaptive behavior (10), the excitability of cortical motoneurones is unimpaired (19) or enhanced (31)

The present investigations did not include studies on the delayed actions of large doses of morphine or its action on the autonomic system, except that observed effects were described The explanations advanced for the effects of morphine on the somatic nervous system may also be applicable to the autonomic centers However, certain special properties which these cells possess by virtue of their functions in maintaining homeostasis, such as their increased activity in response to stimuli (anoxia, hypercapnia, hypoglycemia) which depress the somatic nervous system (32), and the influences of the "buffer nerves", make the total effects of drugs on these structures more difficult of interpretation Likewise, an interpretation of the delayed excitatory effects of morphine must take into account not only compensatory reactions to the primary actions of the drug but also its metabolic effects, which although slight, may be cumulative (e g , anticholinesterase activity, pH changes) In view of these considerations, the hypotheses advanced are of value chiefly as leads for further research and are offered at this time solely from that point of view

SUMMARY AND CONCLUSIONS

1 In the intact cat, small doses of morphine (2 to 5 mgm per kg) caused changes in behavior which appear to represent disintegration of adaptive responses This, in turn, appears to be associated with selective depression rather than with "stimulation" of the nervous system With larger doses (10 to 15 mgm per kg) evidence of delayed stimulant actions ("startle response", muscle twitches, spontaneous running) are superimposed

2 In acute decorticate and hypothalamic cats, 2–5 mgm per kg of morphine depress or abolish the skeletal motor components of "sham rage", and the righting reflexes, the effects being more marked and prolonged in the decorticate animal In both, the forelegs become rigidly extended, and tonic neck and labyrinthine reflexes are often demonstrable With larger doses, the depressant effect is shorter, and may be followed by excitatory effects, especially augmentation of running movements

3 In acute decerebrate cats, extensor rigidity and the labyrinthine reflexes are not altered by small or moderate doses of morphine Delayed excitatory effects, such as alternate rhythmic movements of the forelegs, the "startle" response, and acoustic reflex turning of the head occur earlier than in other preparations an increase in intensity over a period of 4–6 hours

4 In acute and chronic spinal preparations, the responses to nociceptive stimuli (flexor and crossed extensor reflexes) are markedly depressed, while the responses to stretch (knee and ankle jerks) are either not affected or they are slightly augmented by morphine in the dose range studied

5. It is concluded that the action of morphine on the somatic central nervous system of the cat may be resolved into (a) selective depression, with consequent release phenomena, and (b) delayed excitatory effects.

6. Theoretical considerations indicate that the ultimate locus of the depressant action of morphine may be on interneurones. The excitability of motoneurones may be enhanced by a reciprocal mechanism secondary to depression of inhibiting internuncial neurones, or they may be stimulated directly.

7. Species differences in the reaction to morphine probably are due, not to hypothetical differences in the action of the drug on nerve cells, but to (a) difference in neural organization, such as the relative preponderance of sympathetic and parasympathetic, facilitating and inhibiting mechanisms, and (b) differences in the concentration of the alkaloid necessary to produce comparable effects.

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STUDIES ON THE FATE OF MORPHINE SULFURIC ETHER

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The discovery (1, 2) that morphine is excreted chiefly in a bound form led to speculation as to the nature of the conjugate and its possible pharmacologic action. Conjugated morphine has not yet been isolated. This paper concerns the results of studies of a theoretically possible form of bound morphine. Since morphine contains a phenolic hydroxyl, and since many such compounds may be excreted as glucuronides or sulfuric ethers, it would be logical to expect morphine to be excreted as either of these or as a mixture. Oberst (3) has shown that there is a close correlation between the urinary excretion of glucuronic acid and bound morphine. Attempts to isolate bound morphine from urine or to synthesize morphine glucuronide have thus far been unsuccessful. However, morphine sulfuric ether, a bound form of morphine, was synthesized by Stolnikow in 1884 (4). Recently Dr. L. F. Small of the National Institute of Health prepared sufficient amounts of this substance[2] for study. In some instances the potassium salt[3] of this compound was used because of its greater solubility. Urinary excretion studies were made for free and bound morphine in 2 tolerant and 9 non-tolerant dogs, in 1 morphine addict, 10 post addicts and 2 non-addicts. Morphine sulfuric ether was also tested in 2 patients at the 30th hour of the withdrawal period to determine whether or not it relieves abstinence signs or symptoms.

METHODS. Morphine sulfuric ether is very slightly soluble, therefore weighed amounts were dissolved in sufficient boiling isotonic saline to make solutions having a concentration

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[1] Now with Wm. S. Merrell Co., Cincinnati, Ohio.

[2] Morphine sulfuric ether is a white crystalline dihydrate. It is practically insoluble in cold water; its solubility in hot water (95°C.) is 6.5 parts per 1000 parts of water. It is readily soluble in alkali and turns brown in 0.2 M. sodium carbonate after a few days. Its specific rotation $[\alpha]_D^{25}$ in 0.2 M. sodium carbonate is $-84.1°$ ($C = 1.14$). The dry product hydrolyzes slowly over a period of time, about 40 per cent in $2\frac{1}{2}$ years.

[3] *Potassium salt of morphine sulfuric ether:* One gram of morphine sulfuric ether, dissolved in 10 cc. 95 per cent ethyl alcohol, was added with shaking to a warm solution of 0.5 grams of potassium hydroxide in 50 cc. of 95 per cent ethyl alcohol. The solution was at first clear but in a short time became cloudy as crystals of the potassium salt began to appear. Fifty cc. of 60–70° boiling legroin was added and the mixture allowed to cool. The snow-white crystals of the potassium salt of morphine sulfuric ether were removed by filtration and first washed with a small amount of absolute alcohol and then with legroin. The yield was 1 gram or nearly quantitative. The melting point was above 270°. Analysis of the product indicated that it contained no water of crystallization.

of approximately 0 5 mgm /cc   After cooling to about 40° the solution was usually administered intravenously to patients and subcutaneously to dogs   No crystals separated out of solution at this temperature when used immediately   In some of the studies on dogs the potassium salt was given subcutaneously

On the 9 non tolerant dogs, 6 tests were made with morphine sulfuric ether and 3 with the potassium salt   The 2 tolerant dogs received only the potassium salt, morphine excretion prior to substitution is shown for one of these

One morphine addict, stabilized on the minimum amount of morphine sulfate needed to satisfy physical dependence, was given for 9 days approximately 4 times that amount of morphine sulfuric ether and the urinary morphine excretion studied   Later it was learned that the product administered was about ½ morphine, derived from the slow hydrolysis of the ether after its preparation

The 10 post addicts studied were patients who had not been dependent on an opiate for at least six months   In most cases they received only one intravenous dose   One patient (Mc C) received 75 mgm of the drug 4 times daily for 2½ days   The 2 non addicts received the drug subcutaneously

The analyses for both free and bound morphine were made according to previously published methods (1, 2) and the results compared with similar studies made with morphine The results of the analyses are reported in terms of morphine base, and the corresponding morphine base equivalent of the administered dose was calculated to determine the per cent excreted

RESULTS   The results obtained on the excretion of free and bound morphine are shown in table 1   The percentage excretion of free morphine in non tolerant dogs after administration of either morphine sulfuric ether or its potassium salt ranged from 2 5 to 12 5 and for bound from 26 3 to 51 4   The percentage range reported by Gross and Thompson (5) for morphine excretion in dogs after morphine sulfate is 10 to 20 for free and 65 to 85 for bound   The percentage excretion for free morphine in tolerant dogs is about the same as for the non tolerant   The values for bound morphine in the 2 tolerant dogs were about 28 per cent of the administered dose   Thompson and Gross reported 10 to 20 for free and 30 to 60 per cent for bound after morphine sulfate

Too few runs were made on non-addicts to be conclusive, but it appears that excretion is not far different from that in non tolerant dogs   In the post-addicts the percentage range for free morphine was 2 5 to 6 9 and for bound morphine from 28 3 to 51 2   The values do not differ greatly from those reported by Oberst (2) on morphine excretion, except that more values fall in the lower portion of this range   In one addict studied the excretion of both free and bound morphine was decidedly lower than when on morphine sulfate

No outstanding difference was noted in excretion when the drug administered was either morphine sulfuric ether or its potassium salt

DISCUSSION   Morphine sulfuric ether dihydrate apparently decomposes slowly in the dry state over a period of time   Some of the product, about 2½ years old, has as much as 40 per cent free morphine present   The specific rotation in 0 2 M Na_2CO_3 was −71 5°, a decrease of 12 6° from the value found when the product was freshly prepared   The pure product gives a negative test with the phenol reagent (6)   Hydrolysis liberates morphine which then gives a strong positive test with this reagent   Morphine sulfuric ether can be

TABLE 1

The excretion of morphine after administration of morphine sulfuric ether

TEST NUMBER	DRUG USED	NO. SUCCESSIVE DAYS DRUG ADMINISTERED	NO. DAYS TESTS MADE	DOSAGE		MORPHINE (BASE) EXCRETION			
				Total alkaloidal salt administered	Equivalent morphine base	Free		Bound	
				mgm.	*mgm.*	*mgm.*	*per cent*	*mgm.*	*per cent*

A. Non-tolerant Dogs

2SUI	K salt	3	8	540	382	18.3	4.8	190.2	49.8
3SUI	K salt	4	8	1,300	920	37.3	4.1	472.3	51.4
1SUI	K salt	1	3	160	113	8.4	7.1	33.6	29.7
1SUI	MSE.	1	3	160	114	14.1	12.5	37.9	33.3
4SUI	MSE.	2	5	360	256	16.1	6.3	93.5	36.5
5SUI	MSE.	3	7	420	299	21.5	7.2	152.9	51.1
6USP	MSE.	1	5	200	142	5.7	4.0	37.3	26.3
7USP	MSE.	1	4	200	142	5.5	3.9	40.0	28.2
SUSP	MSE.	1	3	100	71	1.8	2.5	19.2	27.0

B. Tolerant dogs

10SUI	M.S.	6	6	660	496	42.4	8.6	197.2	39.8
	K salt	6	6	660	467	19.8	4.2	128.7	27.6
11SUI	K salt	6	9	840	594	33.0	5.6	172.0	29.0

C. Non-addicts

Hi	MSE.	1	1	20	14.2	tr		5.1	35.9
Ob	MSE.	1	1	40	28.4	2.3	7.9	15.7	55.3

D. Post-addicts

Ka	MSE.	1	1	160	113.8	4.4	3.9	44.1	38.8
Ha	MSE.	1	1	80	56.8	2.6	4.6	28.3	49.9
Ka	MSE.	1	2	160	113.8	3.5	3.0	39.7	35.0
As	MSE.	1	1	160	113.8	3.9	3.4	59.1	52.0
Wa	MSE.	1	1	200	142.2	9.9	6.9	66.8	46.9
De	MSE.	1	1	365	259.4	16.2	6.3	89.7	34.6
Mc	MSE.	1	2	160	113.8	7.2	6.8	36.7	32.3
Mc C	MSE.	3	4	750	533.0	19.6	3.7	155.5	28.3
Da	MSE.	1	2	160	113.8	3.5	3.1	61.8	54.2
	MSE.	1	1	200	153.5	4.1	2.5	64.4	41.8
St	M.S.			15					

E. Morphine addict

Th	M.S.	7	7	935	703	38.3	5.5	293.1	41.6
Th	MSE.	9	13	4,420	3,142	91.4	2.9	733.0	23.3

K salt = Potassium salt of morphine sulfuric ether.

MSE. = Morphine sulfuric ether dihydrate.

M.S. = Morphine sulfate pentahydrate.

separated from morphine almost completely by 3 recrystallizations from hot water

After the test was completed on the addict, it was found that the morphine sulfuric ether used contained some free morphine as an impurity   The amount was not ascertained, but is is believed not to have exceeded 20 per cent when the run was made   The compound used in the other studies was tested for free morphine and when present was removed by 3 recrystallizations from hot water after which less than 1 per cent of free morphine was present

The excretion of morphine sulfuric ether and its potassium salt in terms of free and bound morphine is somewhat lower than that following morphine administration   It has not been ascertained whether the bound morphine in urine is the unchanged substance or whether it is the form in which morphine is usually excreted, possibly the glucuronide

The pharmacological activity of this compound apparently is of a lower order than codeine   It does not produce euphoria in man, its pain threshold raising effect as determined by Hardy, Wolff, and Goodell technique (7) is practically absent in man and is questionable in dogs   Knowlton and Gross (8) reported a mild degree of analgesia in dogs with the potassium salt   A 200 mgm dose did not relieve pain in man, neither did 160 to 200 mgm relieve the withdrawal symptoms at the 30th hour as previously reported (9)

The compound given to some patients in amounts of 75 to 160 mgm had a mild hypnotic action   The patients reported a feeling of lassitude and "at ease "   After 2½ days of 75 mgm q i d one patient developed a slight elevation in temperature and claimed to have ' pain in the bones of legs and arms "   The medication was then discontinued and patient recovered from these effects within a few hours

The low pharmacological activity of morphine sulfuric ether, a readily hydrolyzable bound form of morphine, suggests that the bound form of morphine excreted after the administration of a morphine salt might also have little or no pharmacological activity and that the analgesia and euphoria derived from morphine may come from either free morphine or one of its degradation products which as yet defies detection

SUMMARY

The excretion of morphine sulfuric ether and in some instances its potassium salt, has been studied in 9 non tolerant and 2 tolerant dogs, and in 2 non addicts, 1 addict, and 10 post addicts   It is excreted in urine as both free and bound morphine the amounts of which are somewhat lower than that reported for morphine per se

Its pharmacological activity is of a low order, it does not produce euphoria in man nor relieve pain   Neither can it be substituted for morphine in addicts nor does it relieve withdrawal symptoms   Its pain threshold raising effect as determined by Hardy, Wolff, and Goodell technique is practically absent in man and is questionable in dogs

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A STUDY OF THE DIGITOXIN BINDING POWER OF SERUM AND OTHER SOLUBLE TISSUE-PROTEINS OF THE RABBIT

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Oppenheimer (1) observed that the addition of serum to a solution of digitoxin markedly diminishes its toxicity to the isolated frog heart   Hoekstra (2), who confirmed and extended Oppenheimer's observation, claimed that, whereas rabbit serum completely protected the frog heart, frog serum was entirely ineffective   Hoekstra's work was confirmed in part by Brucke (3) who found that frog serum also showed a weak protective action and that this species difference was only of a quantitative nature   The literature on the subject has been reviewed by Lendle (4) and Weese (5)

Hoekstra (2), without presenting any direct evidence (see also Lendle and Pusch (6)) attributed the digitoxin binding power to the serum proteins   The need for further investigation of this problem was indicated by Schliomensum's (7) claim to have isolated from mammalian heart a lipoid fraction capable of binding digitoxin   In our opinion, the only conclusive evidence as to the nature of the digitoxin binding substance in serum would be the isolation of a substance in question in pure form and the demonstration of its digitoxin binding power in vitro   Further, it should be possible to recover the digitoxin quantitatively from this combination   In the present study we have found our criteria to be satisfied by the albumen fraction of serum, insofar as the latter can be regarded a homogeneous substance (8)

METHODS   *Digitoxin*   The criteria for the absolute purity of digitoxin as set forth by Windaus and Freese (9) were fulfilled by a sample of crystalline digitoxin kindly supplied by the firm ' Hoffmann La Roche ''   Recrystallized from ethyl acetate or watery acetone, it had a melting point of 255–256°C   For our experiments a stock solution of 0 5% digitoxin alcohol was prepared, in our test solutions the final concentration of alcohol never exceeded 0 8%, a concentration which was found to be without influence on the isolated frog heart

Preparation of serum and serum proteins   Rabbit blood was collected in a paraffin wax coated cylinder under aseptic conditions and allowed to clot   A paraffin covered balance weight was placed in the cylinder on top of the clot and this was left in the ice box for 24–48 hours after which the serum was siphoned off   Before use the serum was made isotonic for frog tissue by the addition of half its volume of distilled water

Total serum proteins were obtained by saturating a portion of serum with ammonium sulfate and leaving overnight   After filtering and washing with saturated ammonium sulfate the precipitate was dialyzed at 4°C against isotonic Locke s solution

A *Serum globulin* was obtained by mixing serum with one volume of saturated ammonium sulfate solution and letting it stand overnight   After centrifuging the precipitate was washed with half saturated ammonium sulfate and dialyzed as mentioned above   Such a globulin fraction contains cholesterol and phospholipids

B   *Albumen fractions*   The first albumen portion was obtained by treating the mother

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liquor from A. with a solution of 0.1N H_2SO_4 in half saturated ammonium sulfate until permanent turbidity resulted (8). Toluene was added and the solution was left at room temperature for 24–48 hours. The precipate was collected and dialyzed as before. This albumen fraction contained an appreciable amount of cholesterol and phospholipids. The mother liquor from the first albumen fraction was further treated with 0.1 N H_2SO_4 in half saturated ammonium sulfate until permanent turbidity developed; then after standing at room temperature for 24–48 hours, it was collected and dialyzed. This second albumen fraction contains the remainder of the lipids originally found in serum.

The third albumen fraction obtained by further acidification (pH approximately 5) was practically lipid free. When desired all of these albumen fractions were purified by several reprecipitations and in some cases crystalline products were obtained.

Soluble tissue proteins other than serum. Fresh rabbit tissues, namely heart, liver and striated muscle of the hind leg were frozen with liquid air and ground in a chilled mortar to a fine powder. A weighed portion was extracted for three hours at 4°C. with five times its weight of distilled water, while another weighed portion was similarly extracted with 0.26 M phosphate buffer

$$\frac{K_2H\ PO_4}{K\ H_2\ PO_4} = 2:1.$$
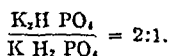
The latter solution was shown by Howe (10) to dissolve the maximum amount of muscle proteins. After filtration the solution was dialyzed. The content of all sera and nitrogen protein solutions was determined by the Kjeldahl method.

Method of testing digitoxin solutions. The Straub isolated frog heart was used. The cannula employed was conical in shape, of such a width that 3 cc. of test fluid rose to a height of 4–5 cm. In order to prevent drying, a wet chamber was placed around the heart.

The frogs used were *Rana temporaria*, obtained from the region of Damascus, Syria. Male and female frogs were used, since sex differences appeared to have no influence on the digitoxin response of the isolated frog heart.

Method for testing the protective action of serum. Eight isolated frog hearts were used to test each solution. The solution remained for 45 minutes in contact with the heart, after which the effect was recorded as (1) no systolic arrest (2) partial arrest, or (3) complete systolic arrest. This method made it possible to differentiate digitoxin concentrations below 1:100,000 with an accuracy of 20–30%.

RESULTS. *Experiments with serum.* It was found that occasionally serum specimens were toxic to the isolated frog heart, consequently control experiments were performed in each case and only non-toxic sera were tested for their protective action.

The final concentration of digitoxin in the test fluid was in each case 1:100,000. This concentration produces *complete systolic arrest within 5–10 minutes* (see fig. 1, *A*). The same concentration of digitoxin in serum showed a diminution in toxicity depending on the season of the year. *Complete protection over the whole test-period of 60–120 minutes* was observed during the cold season, whereas during the warm months, a delay of only 20–30 min. in the systolic arrest could be obtained. Most of our experiments were performed during the cold season.

Table 1 summarizes the results of one of three experiments on serum and the various protein fractions obtained therefrom. There is a positive correlation between protein content of serum and its ability to inactivate digitoxin. Isolated total serum proteins showed qualitatively the same digitoxin binding

power as serum itself. The globulin fraction possessed no protective action whatsoever. The albumen fractions on the other hand exhibited all the protective action inherent in serum itself (see fig 1 B). There was no difference in the protective powers of the various albumen fractions. Crystalline albumen was as effective as the cruder preparations and no consistent qualitative or quantitative differences were observed. As shown in table 1 there is no correlation between lipoid content and protective action.
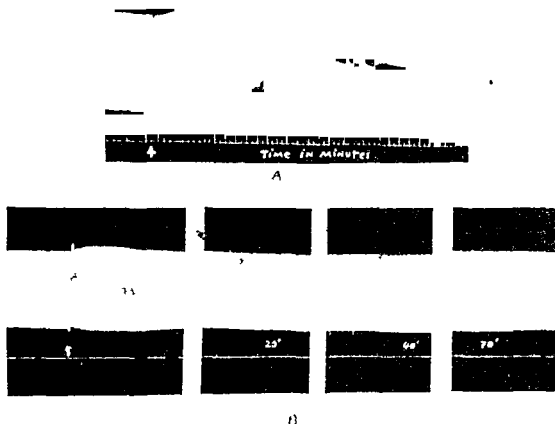
FIG 1 TRACINGS OF ISOLATED FROG HEARTS STRAUB TECHNIC

A 3 cc of a 1 100 000 solution of digitoxin in Locke solution introduced at arrow resulting in complete systolic arrest (control)

B 3 cc of a 1 100 000 solution of digitoxin in a 2% crystalline rabbit serum albumen solution introduced at arrow showing complete inactivation of digitoxin

Soluble proteins of heart liver and striated muscle obtained as described under methods were tested for their ability to inactivate digitoxin with entirely negative results in all experiments performed.

The recovery of digitoxin after its combination with serum proteins Hitherto the digitoxin binding power of a substance was measured by its ability to protect the isolated frog's heart against a toxic dose of digitoxin. A more direct method was developed based on the diffusibility of digitoxin through a cellophane membrane[1]. Thus if a 1 33 000 digitoxin solution in Locke's solution is dialyzed

[1] Cellophane bags used were visking cellulose sausage casings size 32/32 kindly supplied by the Visking Corporation Chicago

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against two volumes of Locke solution at 4°C. for 48 hours, the digitoxin concentration inside and outside the bag are the same and amount to 1:100,000 as determined on the isolated frog heart. Identical results were obtained if 1:33,000 digitoxin in 2–3% rabbit serum globulin is dialyzed in a similar manner showing that serum globulin does not combine with digitoxin (fig. 2, *A*). The addition of as little as 2% serum albumen to a 1:33,000 digitoxin solution is sufficient to prevent the diffusion of the greater amount of digitoxin as evidenced

TABLE 1

Serum 12.   Dec. 15–22, 1940.   Pooled serum from three rabbits

	SOLUTION	PROTEIN	CHOLES-TEROL	PHOS HATIDE	NUMBER OF HEARTS TESTED	HEARTS SHOW-ING NO SYS-TOLIC ARREST	HEARTS SHOW-ING PARTIAL ARREST	HEARTS SHOW-ING COM-PLETE SYS-TOLIC ARREST
		gm. %	*mg.* %					
	Control, digitoxin 1:100,000				8	None	1	7
Whole serum	Serum 12	1.0	9.4	Present	8	8	None	None
	Serum 12	0.5	4.7	Present	8	6	2	None
	Serum 12	0.25	2.35	Present	8	4	2	2
	Serum 12	0.10	0.95	Present	8	None	5	3
Globulin fraction	Globulin	3.0	45 0	Present	8	None	2	6
1st albumen fraction	Albumen I, pH 5.5	0.25	17.3	Present	8	6	2	None
	Albumen I, pH 5.5	0.15	10.3	Present	8	2	4	2
	Albumen I, pH 5.5	0 075	5.15	Present	8	None	4	4
2nd albumen fraction	Albumen II, pH 5.3	0 25	4 0	Trace	8	6	1	1
	Albumen II, pH 5.3	0.15	2.4	Trace	8	2	3	3
	Albumen II, pH 5.3	0 075	1.2	Trace	8	None	6	2
3rd albumen fraction crystalline	Albumen III, pH 5.1	0 25	None	None	8	6	2	None
	Albumen III, pH 5.1	0.15	None	None	8	1	5	2
	Albumen III, pH 5.1	0.075	None	None	8	None	4	4

by the fact that both outside and inside fluids are practically non-toxic to the heart (fig. 2, *B*).

The protective action of serum proteins has been ascribed to their ability to bind digitoxin. Although this hypothesis seemed to be the most plausible, the alternative and less likely explanation, the destruction of digitoxin by serum proteins, had to be ruled out.

Digitoxin was added to a measured serum or serum albumen solution to give a concentration of 1:33,000. This solution, which is practically non-toxic to the isolated heart, was treated with two volumes of cold alcohol with the object

of denaturing the serum proteins. After standing 15 minutes the precipitate was filtered off. An adequate portion of the filtrate was evaporated to one-tenth its volume in a vacuum desicator over calcium chloride. To this material
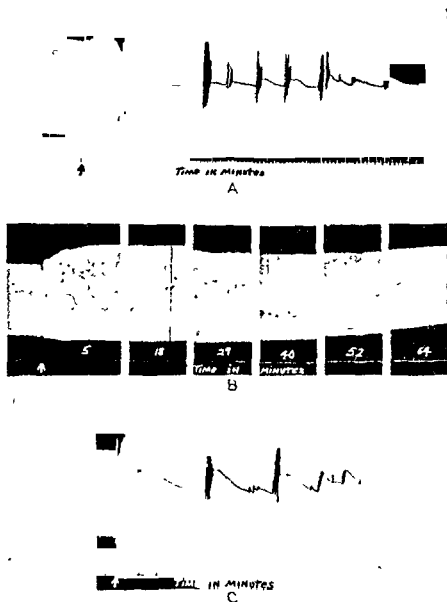
FIG. 2 .TRACINGS OF ISOLATED FROG HEARTS, STRAUB TECHNIC DIALYSIS EXPERIMENTS

A, 1:33,000 digitoxin in 2% rabbit serum globulin dialyzed against two volumes of Locke's ... on of digitoxin

... halysate intro-

... cc. of alcohol ... digitoxin.

enough frog isotonic Locke's solution was added to make the digitoxin concentration 1:100,000. After warming for a few minutes at 60°C. the solution was tested on the isolated frog heart. This solution had the activity of a 1:100,000 digitoxin solution (fig. 2, *C*), thus indicating that digitoxin can be recovered by

denaturing the protein to which it is bound. Control experiments were performed by subjecting serum or serum albumen solutions to the same procedure. The resulting test solution was found to be non-toxic to the Straub heart. We were unable to confirm Hoekstra's claim (2) to have liberated the digitoxin from its protein combination by treatment with alkali to pH 8.3, with saponin, or with bile salts in concentrations which were non-toxic to the isolated frog heart.

SUMMARY

Rabbit serum protects the isolated frog heart against digitoxin, this protective action being solely attributable to the albumen fraction. Furthermore this protective action is directly proportional to the protein content of the solution. This quantitative relationship, as well as the results of our dialysis experiments, indicate that the mechanism of the protective action lies in a combination of digitoxin with serum albumen, which can be split and the digitoxin recovered quantitatively by treatment with alcohol. Rabbit serum globulin and serum lipoids, as well as the soluble proteins of the heart, liver and striated muscle, show no protective action against digitoxin.

The manuscript for this paper was prepared early in 1941. Due to war conditions it has not been possible to publish it promptly. Meanwhile a number of papers by Lendle and al. (11, 12, 13) have come to our notice dealing with the same subject. Since these articles are not available to us, we are in no position to comment on them.

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THE EFFECT OF PROPAMIDINE AND CERTAIN OTHER DIAMIDINES ON THE OXIDATION OF VARIOUS SUBSTRATES BY E COLI[1]

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Bactericidal or bacteriostatic drugs may affect the metabolism of bacteria in various ways The sulfa drugs apparently interfere with certain anabolic reactions involving p aminobenzoic acid, methionine and other compounds Gramicidin (1) may, depending on the conditions, increase or decrease the oxygen uptake of bacteria Certain bactericidal aromatic iodine compounds (2) increase the oxygen uptake of the tubercle bacillus while others decrease it In aerobes, interference with the oxidative metabolism by certain bactericidal drugs may lead to an increase or decrease of their oxygen uptake depending on the experimental conditions, the concentration of the drug and possibly other factors such as the age of the culture, and the strain used The recent demonstration (3) that certain diamidines inhibit the growth of bacteria made it of interest to investigate the mechanism of this inhibition Preliminary results (4) indicated that the oxidative metabolism was affected by these drugs and the following is a more detailed study of this effect

EXPERIMENTAL

E coli No 6522 of the American Type Culture Collection was grown for 18 hours at 37° in 200 cc of a medium containing 2% proteose peptone, 0 6% NaCl and 200 mg % glucose at pH 7 8 The bacteria were then centrifuged and washed once with water containing a few cc of 0 05 M phosphate buffer pH 7 8 They were then suspended in phosphate buffer, placed in Warburg vessels with various substrates and the oxygen uptake measured in the presence and absence of propamidine and certain other diamidines The oxygen uptake of the bacteria without the addition of substrates was very small, i e , 15 to 20 cmm of oxygen per hour Previous results (4) indicated that the oxidation of nitrogen containing compounds was inhibited by concentrations of propamidine which had little or no effect on the oxidation of glucose or pyruvic acid This has been confirmed and the results are shown in table 1 The table also shows that it is necessary to incubate the drug with the bacteria before the addition of the substrate This latent period probably represents the time necessary for the penetration of the drug and occurs also in experiments on the inhibition of growth (5) Nephelometric readings as well as evidence from the shape of the curves showed that no growth occurred in the Warburg vessels

Both isomers of alanine are oxidized by this strain of E coli at approximately equal rates and propamidine inhibits their oxidation equally That it is the

oxidative deamination of alanine which is inhibited is proved by the fact that the oxidation of pyruvic acid is not affected under the same conditions and that ammonia production is less in the suspensions containing the drug than in the controls. On the other hand the oxidation of serine is markedly accelerated by concentrations of propamidine which inhibit the oxidation of alanine. This is also shown in table 1. Ammonia determinations made during the period of maximal acceleration show that propamidine increases the formation of ammonia in the suspensions, which suggests that it is the oxidative deamination of serine that is accelerated. Higher concentrations of the drug will inhibit the oxidation of serine. Acceleration of the oxidation of alanine was never ob-

TABLE 1

The effect of the time of incubation (before the addition of substrate) on the action of 2.5 × 10⁻⁵M propamidine on the oxidation of 1.0 mg. l + alanine and 2.0 mg. dl-serine. 37°. pH 7.8

The effect of propamidine on the oxidation of 1.0 mg. d-alanine, 1.0 mg. glycine, 1.0 mg pyruvic acid and 1.0 mg. glucose is also shown.

TIME	1 HOUR INCUBATION						2 HOUR INCUBATION					
	1 + ala-nine	1 + ala-nine + propa-midine	Inhibi-tion	dl-serine	dl-serine + pro-pami-dine	Acceler-ation	1 + ala-nine	1 + ala-nine + propa-midine	Inhibi-tion	dl-serine	dl-ser-ine + propa-midine	Acceler-ation
min.	cmm.	cmm.	%	cmm.	cmm.	%	cmm.	cmm.	%	cmm.	cmm.	%
30	47	39	17	86	110	28	31	21	32	75	119	59
60	116	96	18	160	207	29	37	54	44	175	262	49
90	195	153	21	222	253	14	168	91	46	270	387	43
120	300	215	28	321	340	6	257	129	50	361	451	25

TIME	2 HOUR INCUBATION											
	d-ala-nine	d-ala-nine + propa-midine	Inhibi-tion	pyru-vic acid	pyruvic acid + propa-midine	Inhibi-tion	glucose	glucose + propa-midine	Inhibi-tion	glycine	gly-cine + propa-midine	Inhibi-tion
min.	cmm.	cmm.	%	cmm.	cmm.	%	cmm.	cmm.	%	cmm.	cmm.	%
30	22	15	32	139	134	4	206	204	0	17	17	0
60	59	34	42	228	221	3	381	382	0	34	32	0
90	105	55	47	296	281	5	437	439	0	53	51	0
120	155	74	52	319	321	0	464	467	0	67	68	0

served although the amount of the drug was varied almost one hundred fold (table 2). Concentrations of propamidine which inhibit the oxidation of alanine and accelerate that of serine have no effect on the oxidation of glycine which is only inhibited at relatively high concentrations. These facts show that a drug even under the same experimental conditions can either accelerate or depress or have no effect on the oxidation of closely related substances. The oxidation of proline is always inhibited by propamidine and therefore behaves like alanine, whereas the oxidation of asparagine, like serine, is either inhibited or accelerated depending on the concentration of drug. This strain of E. coli does not oxidize phenylalanine, leucine, or methionine.

Table 2 shows that the inhibition of the oxidation of alanine increases with the increase in drug concentration but not proportionally This indicates that the rate of penetration as well as the inhibition of the catalyst responsible for the oxidation of the amino acid may vary with the concentration of the drug On the other hand the number of bacteria has a definite effect on both the

TABLE 2

The effect of different concentrations of propamidine on the oxidation of 1 0 mg l + alanine at 37°   pH 7 8

All concentrations of the drug were shaken 2 hours with the E coli before the addition of the alanine   The final volume in each vessel was 2 0 cc

	MG PROPAMIDINE																
TIME	0 00	0 005	Inhibition	0 01	Inhibition	0 02	Inhibition	0 04	Inhibition	0 08	Inhibition	0 1	Inhibition	0 2	Inhibition	0 4	Inhibition
min	cmm	cmm	%	cmm	%	cmm	%	cmm	%	cmm	%	cmm	%	cmm	%	cmm	%
30	44	38	14	33	25	30	32	32	27	30	32	28	36	7	84	0	100
60	109	94	14	82	25	71	35	69	37	63	42	57	47	14	87	0	100
90	192	161	16	142	26	125	35	113	41	102	47	94	51	27	86	0	100
120	295	231	22	201	32	176	40	156	47	137	54	127	57	35	88	0	100
150	413	307	26	262	37	228	45	200	52	171	56	157	64	43	90	0	100

TABLE 3

The effect of the relative concentration of bacteria and propamidine on the oxidation of 2 0 mg dl serine and 1 0 mg l + alanine   37°   pH 7 8

The volume in each vessel was 2 0 cc   The propamidine was shaken for 2 hours with the E coli before the addition of the amino acids

	0 25 CC SUSPENSION OF E COLI								0 5 CC SUSPENSION OF E COLI							
TIME	alanine	alanine + 0 02 mg propamidine	inhibition	serine	serine + 0 02 mg propamidine	acceleration	serine + 0 1 mg propamidine	acceleration	alanine	alanine + 0 02 mg propamidine	inhibition	serine	serine + 0 02 mg propamidine	acceleration	serine + 0 1 mg propamidine	acceleration
min	cmm	cmm	%	cm n	cmm	%	cmm	%	cmm	cmm	%	cmm	cmm	%	cmm	%
30	30	18	40	43	82	91	67	55	57	43	25	83	160	93	141	70
60	87	48	45	108	206	91	169	56	152	111	27	209	299	43	272	30
90	159	76	52	189	288	52	234	24	285	187	34	336	347	3	333	0
120	243	112	54	263	323	23	282	7	419	261	34	390	384	0	357	−11

inhibition of the alanine and acceleration of the serine oxidation   This is shown in table 3 and indicates that a certain number of molecules must be fixed by each cell for a certain effect to be obtained   Table 4 shows the effect of hydrogen ion concentration   At pH 6 7 propamidine has much less effect on the serine and alanine oxidations than at pH 7 8 which suggests that the unionized molecule penetrates more readily   Finally table 5 shows the effect of three other diami-

dines on the alanine and serine oxidations. Pentamidine and stilbamidine are almost as effective as propamidine, but phenamidine is almost without action in the concentration used. Evidently the number of carbon atoms between the two rings is important for the action of these compounds.

TABLE 4

The effect of hydrogen ion concentration on the action of propamidine on the oxidation of 1.0 mg. d + alanine and 2.0 mg. dl-serine. 37°

The propamidine was shaken for 2 hours with the E. coli before the addition of the amino acids

TIME	pH 6.7						pH 7.8					
	alanine	ala-nine + propa-midine	inhibi-tion	serine	serine + propa-midine	acceler-ation	ala-nine	ala-nine + propa-midine	inhibi-tion	serine	serine + propa-midine	acceler-ation
min.	cmm.	cmm.	%	cmm.	cmm.	%	cmm.	cmm.	%	cmm.	cmm.	%
30	15	12	20	23	30	30	17	8	53	32	56	75
60	39	36	8	56	67	20	41	19	53	72	109	52
90	72	64	11	95	103	8	79	30	62	120	160	33
120	109	95	13	128	132	3	120	43	64	161	204	27

TABLE 5

The effect of 0.02 mg. each of phenamidine, stilbamidine and pentamidine compared to the effect of 0.02 mg. propamidine on the oxidation of 1.0 mg. l + alanine and 2.0 mg. dl-serine. 37°. pH 7.8

The volume in each vessel was 2.0 cc. The drugs were shaken 2 hours with the E. coli before the addition of the amino acids.

TIME	ALANINE	+ PROPA-MIDINE	INHIBI-TION	+ STILBA-MIDINE	INHIBI-TION	+ PENTA-MIDINE	INHIBI-TION	+ PHENA-MIDINE	INHIBI-TION
min.	cmm.	cmm.	%	cmm.	%	cmm.	%	cmm.	%
30	44	29	34	31	30	31	30	46	0
60	112	71	37	78	31	75	33	114	0
90	199	125	37	134	33	129	35	199	0
120	302	175	42	189	37	186	39	285	6
150	427	229	46	243	40	248	42	381	11
	SERINE		ACCELERA-TION		ACCELERA-TION		ACCELERA-TION		ACCELERA-TION
	cmm.	cmm.	%	cmm.	%	cmm.	%	cmm.	%
30	34	71	108	62	82	55	62	42	23
60	79	161	102	128	62	127	61	96	21
90	113	214	98	171	51	168	48	135	19
120	156	251	61	216	38	199	28	183	17
150	199	276	39	241	21	221	11	218	8

Kohn (5) found that incubation of propamidine with E. coli in the presence of glucose increased the subsequent inhibition of the growth of the bacteria by the drug. It was therefore of interest to determine whether the presence of a small amount of glucose during the 2 hours incubation period would increase the

subsequent inhibition of the oxidation of alanine   In five such experiments such an increase was observed four times   Thus in the absence of glucose the average initial inhibition of the alanine oxidation was 20% whereas when 0 5 mg glucose was present during the incubation period the average inhibition was 33%   The glucose was completely oxidized before the addition of alanine   Glucose or the oxidation products apparently increase the penetration of propamidine into the cell

DISCUSSION

Since propamidine interferes with the oxidation of certain substances by E coli in concentrations comparable to those which inhibit growth it is reasonable to conclude that growth is inhibited because of the disturbance in the oxidative metabolism   The action of the drug appears highly specific, the oxidation of some amino acids being inhibited, others accelerated, and some unaffected   The fact that the oxidations of glucose and pyruvic acid are not affected by concentrations that affect the oxidation of amino acids indicates that the cytochrome system is not involved   This assumption is supported by the fact that the oxidation of succinic acid is not inhibited by the drug under identical conditions

SUMMARY

1 Propamidine inhibits the oxidation of l proline and of both isomers of alanine by E coli   Under the same conditions it accelerates the oxidation of serine and asparagine although higher concentrations inhibit   The oxidation of glycine is inhibited but only by relatively high concentrations

2 The action of the drug requires an incubation period of at least 2 hours The presence of glucose during this incubation period increases the subsequent effect of the drug

3 The drug concentration relative to the number of bacteria present determines its effect on the oxidation of the amino acids

4 The oxidation of glucose, pyruvic acid and succinate is not affected by concentrations which inhibit or accelerate the oxidation of the amino acids

5 Propamidine is much more effective at pH 7 8 than at pH 6 7

6 In comparable concentrations pentamidine and stilbamidine are only slightly less active than propamidine but phenamidine is almost entirely inactive

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ACTION OF ANTICHOLINESTERASES ON THE MOTILITY OF THE EXTRINSICALLY DENERVATED INTESTINE IN SITU[1]

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In a previous study (1), using Thiry fistulae in unanesthetized dogs, it was observed that vagotomy and sympathectomy did not reduce the inhibitory action of atropine on intestinal motility Postganglionic sympathectomy resulted in some increase in the inhibitory effect of atropine In the present study the actions of physostigmine and prostigmine on the denervated intestine ' in situ ' and the interactions of atropine, physostigmine and choline derivatives have been recorded

METHODS Intestinal motility was recorded by balloon methods from extrinsically denervated Thiry fistulae made from the upper jejunum In some cases a mercury manometer was used for recording A balloon volume of 6 to 8 cubic centimeters and balloon length of 5 centimeters was used In other cases volume changes were recorded with a constant pressure of 20–25 centimeters of water and a balloon length of 5 centimeters The intestinal segments were doubly denervated by cutting the nerves in the mesentery and along the blood vessels and painting the vessels with carbolic acid in animals already having the vagi sectioned at the level of the esophagus and the splanchnic nerves cut and the lumbar sympathetic chains removed Completeness of the denervation was indicated by failure to elicit the intestino-intestinal reflexes and by the absence of a pain response to high pressure The effects of the drugs were studied within a few days to several weeks after the mesenteric denervation

RESULTS I *Sensitivity to choline derivatives during the period of inhibition of the denervated intestine produced by atropine* A dilution of carbaminoylbeta methylcholine was determined which was barely sufficient to produce a definite increase in intestinal motility when injected intravenously from a motor driven syringe at a rate between 1 to 4 cc per minute The effect of the test dose was determined before and during the inhibition of the denervated intestine produced by intravenous injection of 0 02 mgm of atropine per kilogram Although this dose of atropine produces only partial inhibition of the intestine it diminishes the response to carbaminoylbetamethylcholine Previous analysis of the effect of acetylcholine on the intestine (2) has shown that intravenous injection of this drug into normal dogs, in doses of 0 025 to 0 200 mgm per kgm , causes a brief sharp contraction or spasm which is a result of the direct action of acetylcholine on intestinal smooth muscle This contraction is followed by prolonged inhibition which is the result of sympathomimetic substances liberated because of reflex activation of the sympatho-adrenal system and by other mechanisms

Whereas, 0.025 to 0.05 mgm. of acetylcholine per kgm. intravenously is sufficient to produce a brief spasm of the denervated intestine in unmedicated animals, a dose many times as great has no effect on the intestine during the period of complete inhibition produced by intravenous injection of 0.065 mgm. of atropine per kgm.   The recovery of sensitivity of the intestine to the excitatory action of acetylcholine following atropinization parallels the return of intestinal motility.

The results indicate that a dose of atropine sufficient to inhibit intestinal motility is also sufficient to decrease the stimulatory action of choline derivatives reaching the intestine via the blood stream.

Therefore, if acetylcholine is being produced in the intestinal wall and any of it is acting after local diffusion its effectiveness would be diminished by any dosage of atropine sufficient to affect intestinal motility.

II. *Effect of physostigmine and prostigmine on the motility of the denervated intestine "in situ."*   Both of these drugs (physostigmine salicylate, Merck and Co. and prostigmine methylsulfate, Hoffman La Roche) exert a stimulatory action on the motility of the denervated intestine when given in doses of 0.01 to 0.05 mgm. per kilogram.   The former amount is near the minimal effective dose. The intestine shows a gradual reduction in "diastolic" volume and may develop a spasm which allows little or no filling at a hydrostatic pressure of 25 cm. of water.   The response is illustrated in figure 1.   This response has been recorded repeatedly in five animals.

The response of the denervated intestine to acetylcholine before and after administration of prostigmine was recorded repeatedly in two animals.   It was observed that an amount of prostigmine barely sufficient to have a stimulant action on the motility of the denervated intestine invariably results in increased duration of the brief spasm of the intestine produced by intravenous injection of a test dose, of 0.025 to 0.150 mgm. of acetylcholine per kgm.   In some instances the duration of the spasm is tripled.   It may be concluded that an amount of prostigmine sufficient to have any stimulatory action on the motility of the denervated intestine is also sufficient to potentiate the action of any acetylcholine being produced locally in the intestine and diffusing to its site of action or arriving by the blood stream.

III. *The effect of atropine on the action of physostigmine and prostigmine on the denervated intestine.*   The increased tonus or spasm of the denervated intestine produced by physostigmine or prostigmine in mild doses may be counteracted and subnormal tonus and motility produced by injection of atropine.   This result is illustrated in figure 1 and figure 2.   The dose of atropine needed is small and is similar to that required to inhibit the intestine in the absence of prostigmine and physostigmine.   The degree of response to a mild test dose of physostigmine *is lowered or eliminated* by a dose of atropine sufficient to inhibit the motility of the denervated intestine, but motility may be induced by injection of more physostigmine.   This result is illustrated in figure 2.

The actions of physostigmine and prostigmine on the denervated intestine "in situ" are qualitatively and quantitatively similar to their actions on the
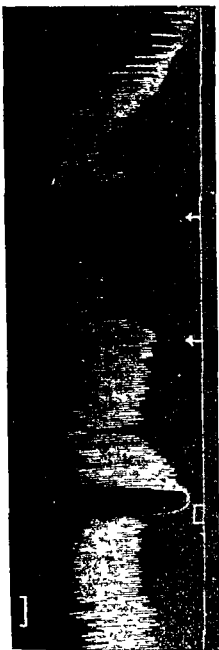
Fig 1 Record of Volume Changes in the Presence of a Constant Pressure of 25 Cm of Water, from a Denervated Intestinal Segment

The first mark indicates time of injection of adrenalin 1 part in 100,000 at a rate of one cc per minute The first arrow indicates time of injection of 0 12 mgm of prostigmine intravenously The second arrow indicates the time of injection of 0 6 mgm of atropine subcutaneously Mark in upper left corner indicates duration of one minute Dog weight 10 kilo

Fig 2 Pressure Record from Denervated Intestinal Segment

Marks indicate the intravenous injections as follows (1) 0 12 mgm of prostigmine, (2) 0 31 mgm of atropine and (3) 0 12 mgm of prostigmine Duration of one minute is indicated by the mark in the upper left hand corner Dog weight, 10 kilo

innervated intestine.   The results may be explained by postulating that these drugs exert an excitatory action on intestinal motility exclusive of anti-cholinesterase action; or the results may be explained in terms of the anti-cholinesterase action of these drugs.   In the latter case it would be possible that the acetylcholine is being produced in the intestine by intrinsic mechanisms or, less likely, the blood level of acetylcholine is being increased.

CONCLUSIONS

Prostigmine and physostigmine exert their characteristic excitatory action on the motility of the intestine "in situ" in the absence of the extrinsic nerves.

Atropine reduces or prevents the excitatory actions of prostigmine and physostigmine on the motility of the extrinsically denervated intestine "in situ."

The minimal dose of the anti-cholinesterases required to stimulate the denervated intestine is sufficient to potentiate the excitatory action of acetylcholine on the motility of the denervated intestine.

The minimal dose of atropine required to inhibit the motility of the denervated intestine is sufficient to reduce or eliminate the excitatory action of acetylcholine on the motility of the denervated intestine.

The results may be explained by postulating that physostigmine and prostigmine exert an excitatory action on intestinal motility exclusive of their anti-cholinesterase action.   On the other hand the results may be explained in terms of the anti-cholinesterase action of these drugs, if it is assumed that there is continuous production of acetylcholine in the denervated intestine at a rate sufficient to stimulate motility.   Finally, the possibility that there is an increase in the acetylcholine content of the arterial blood reaching the intestine, even though the dose of anti-cholinesterase is small, has not been ruled out.

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THE HYPOTHALAMIC CONTROL OF ASPIRIN ANTIPYRESIS IN THE MONKEY[1]

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The regulation of a balancing between heat production and heat dissipation in maintaining normal body temperature and the disturbance in the balance in fever have recently been discussed fully by Du Bois (1)   The antipyretic action of drugs has been attributed to an increase in heat loss rather than to decrease in heat production   Barbour and his co workers (2, 3) have studied intensively factors involved in temperature regulation, particularly changes in blood concentrations   In monkeys, subjected to warmed environment or experimentally induced fever, they have shown that the antipyretic action of aspirin involves a hydration of the blood, leading to increased heat radiation and sweating

It is generally accepted that the hypothalamic region of the brain plays an essential role in the heat production and dissipation mechanisms   Since sweating is a constant effect in the antipyretic action of salicylates, the work reported here was a study of aspirin effects, particularly, in monkeys with hypothalamic lesions   It was hoped that the experiment would throw more light on the site of the regulating areas   The procedures adopted follow

PROCEDURES   In the experiments fourteen monkeys (*Macaca mulatta*) were employed They ranged in weight from 3 8 to 6 5 kgm and had been used in the previous work   In five of them numbers 12 14, 15 16 and 17, hypothalamic lesions were produced, they were com pared with the unoperated animals under the following experimental conditions

   Group C—Normal monkeys in prone position on padded board at control room tem perature of 23°C (±5°C) receiving 100 mgm /kgm of aspirin[3] by stomach tube (15 observations)

   Group Co—Monkeys with hypothalamic lesions in same position and environment as those in Group C and also receiving 100 mgm /kgm of aspirin by stomach tube (5 observations)

   Group D—Normal monkeys tied in prone position on padded board first at a room temperature of 20–23°C , after a short control period they were placed in a room at 40°C dry air, without administration of any drug (10 observations)

   Group Do—Monkeys with hypothalamic lesions in the same position and environ ment as in D (5 observations)

   Group G—Febrile monkeys tied in prone position on padded board in room at 23°C (±5°C) and given 100 mgm /kgm of aspirin at the fever plateau (16 observations)

   Group Go—Febrile monkeys with hypothalamic lesions in the same position and environment as in G and also given 100 mgm /kgm of aspirin at the fever plateau (10 observations)

In the present experiments we used exclusively as pyrogenic substance 5 cc./kgm. of a 25% sterile yeast suspension in water injected subcutaneously. Following the injection the animal was returned to the cage and left with freedom of movement. By the end of three and a half hours the maximum febrile response appeared. At this point observations were again made with the animal tied on the padded board. The rectal temperature was taken each ten minutes at the same time that the respiratory rate was recorded. The quantitative method of sweating estimation during ten minute periods using the same area of the left hand of the monkey, has been previously described (3). The normal values of the sensible and insensible perspiration of this constant area range between 0.04 and 0.07 gm. (in water) per ten minute period. This method permits the estimation of differences of 0.0001 gm. (water).

Electrolytic lesions of the hypothalamus were made with the use of the Horsley-Clarke instrument with a direct current of 3 M.A. for 30 sec. employing the following coordinates: A 15½, L 3, H-3, H-1; L 1½, H-4, H-2, H 0; R 1½, H-4, H-2, H 0; R 3, H-3, H-1. Subsequent histological examination demonstrated the presence of bilateral lesions at the level of the caudal border of the chiasma involving the anterior hypothalamic area and the anterior

PHOTOMICROGRAPH 1. Monkey #14

portion of the lateral hypothalamic area, including the region of the median forebrain bundle. In one of the animals (#15) the lesions were asymmetrical; on the right (see photomicrograph #2) the damage included the medial portion of the internal capsule (which may explain some paralysis of the posterior limbs observed). In the animal #12 (see photomicrograph #3) the inconstant results obtained were due to the fact that although lesions were placed in the right side in the dorsal part of the anterior hypothalamus, the lesions on the left appeared too high. At autopsy this animal also showed symmetrical focal lesions of the cortex probably caused by yeast emboli.

It is necessary to point out the order in which the experiments were performed. Usually in this kind of research, observation of the biological response in the same animal under different experimental conditions, must be carried out according to a latin square (5 × 4). However, the experiments in the animals with hypothalamic lesions were performed according to the following pattern:

$$
\begin{array}{ccccc}
Co & Do & Co & Do & Co \\
Do & Co & Do & Co & Do \\
Go_1 & Go_1 & Go_1 & Go_1 & Go_1 \\
Go_2 & Go_2 & Go_2 & Go_2 & Go_2 \\
\end{array}
$$

This particular sequence avoids any conditioned reflex that could appear in the short period of the experiments (25 days between the operation and the autopsy of animals) If the following latin square (5 × 4) had been used

$$Co \quad Go_1 \quad Do \quad Go \quad Co$$
$$Do \quad Co \quad Go_2 \quad Co \quad Go_1$$
$$Go_1 \quad Do \quad Co \quad Go_1 \quad Do \qquad Go_1 = Go_2$$
$$Go_2 \quad Go_2 \quad Go_1 \quad Do \quad Go_2$$
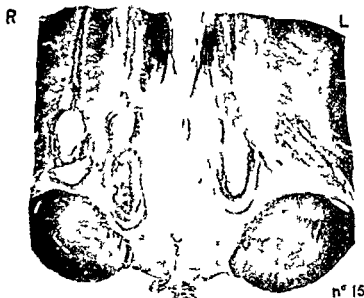
PHOTOMICROGRAPH 2 Monkey #15
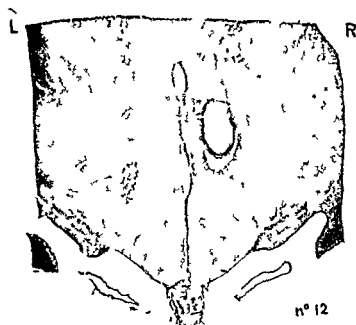
PHOTOMICROGRAPH 3 Monkey #12

any control experiment performed in animals which were submitted to fever condition Group Go_1 would not be valid because the aspirin antipyretic action persists beyond the period of actual fever and would alter the results

100 mgm /kgm of aspirin were given in suspension of 25 cc of water by stomach tube

as indicated in the tables Co and Go. In the G and Go groups the animal received the aspirin at the onset of the fever plateau. Its effect was observed fifteen minutes after administration.

As a technique for determining the plasma specific gravity the Falling Drop method of Barbour and Hamilton was employed (4). Chlorides were determined according to the Van Slyke and Sendroy method (5). The study of the lesions in the hypothalamus was made using sections stained by the Weil technique.

RESULTS. *Temperature.* During 48 to 72 hours after lesions had been made in the anterior and lateral regions of the hypothalmus, the rectal temperature of animals with freedom of movement in the cage fell from an average of 39.0°C. (30 observations) with an average diurnal variation of $\pm$ .2°C. to an average of

TABLE Co
Operated monkeys in control room receiving aspirin at ($\blacktriangle$)
(Average of 5 experiments on 5 monkeys)

	TIME (MINUTES)								
	10	20	30	40	($\blacktriangle$)	50	60	70	80
Temperature (°C.).......	38.4	38.5	38.4	38.4		38.3	38.2	38.2	38.0
Respirations per minute ..	48	48	46	48		46	47	47	47
Sweat (gm. of H_2O) .. .	.062	.057	.048	.050		.045	.047	.040	.042
Plasma gravity		1.0277						1.0276	
Plasma chloride (m.eq/l.)..		110						108	

TABLE Do
Operated monkeys in hot room (40°C.) at ($\blacksquare$)
(Average of 5 experiments on 5 monkeys)

	TIME (MINUTES)												
	10	20	30	40	($\blacksquare$)	50	60	70	80	90	100	120	130
Temperature (°C)	37.5	37.5	37.3	37.0		36.8	36.8	37.1	37.5	38.1	38.6	39.2	39.7
Respirations per minute	38	39	39	37		38	39	44	45	42	44	46	48
Sweat (gm. of H_2O)		.066	.052	.040		.040	.037	.018	.020	.034	.045	.046	.054
Plasma gravity	1.0303						1.0298					1.0295	
Plasma chloride (m eq/l.)	109						108					117	

34.8°C. (10 observations) with individual variations from 34.0°C to 35.3°C. In successive days there appeared a progressive rise in the rectal temperature until at the end of two weeks the average in 30 observations was 37.3°C. Considerable individual differences were observed on different days in response to the changes in environmental temperature. Even several weeks after the operation, the animals' body temperature was quite variable. In the Co group receiving aspirin while tied down at a room temperature of 20°C. there occurred a small fall of temperature which paralleled the fall that occurred before the operation (about .3°C. in an hour). In the Group Do the rise of rectal temperature in response to the hot environment (dry air 40°C.) was more rapid in the *animals* with anterior hypothalamic lesions (0.5°C. each ten minutes) than in the same

TABLE 60

Operated febrile monkeys in control room receiving pyrogenic injection at (X) and aspirin at (▲)

(Average of 10 experiments in 5 monkeys)

	10	20	30	40	(X)	50	60	120	180	240	300	340	350	360	370	(▲)	380	390	400
Temperature (°C)	38.5	38.4	38.3	38.3		38.2	38.0	37.7	38.5	39.1	39.3	39.4	39.2	39.0	38.9		38.9	38.8	38.7
Respirations per minute	41	41	41	40		40	40					52	52	52	52		55	55	56
Sweat (gm. of H₂O)	.062	.055	.050	.048		.044	.042					.055	.048	.044	.037		.037	.034	.033
Plasma gravity			1.0294									1.0272					1.0269		
Plasma chloride (m eq/l)			103									103					106		

TIME (MINUTES)

present. Ranson rarely found it after this type of lesion but noted it frequently after posterior lesions. In monkey # 12 with a single lesion in the dorsal hypothalamus on the right side and a thalamic lesion on the left, hypothermia did not occur. In this monkey there were irregular responses to sweating provoked by heat or fever. After bilateral lesions, Ranson did not find sweating in some monkeys placed in a hot box temperature of 102° to 104°F. In our operated monkeys # 14, 15, 16 and 17, there was an absence of sweat production in response to both environmental heat and fever.

CONCLUSIONS

A marked lability of temperature regulation appeared in monkeys after lesions in the anterior and anterolateral hypothalamus. The unstable character of temperature control was manifested in febrile animals and in response to a hot environment. This state was temporary, however, as the animals progressively regained their regulatory responses.

The hypothalamic lesions did not alter the rate of decline of fever produced by yeast injection or following aspirin administration.

The lesions altered the respiratory response to environmental warming and fever. In general the three series of operated animals tended to show a more rapid respiratory rate than did the unoperated ones. In fever in the unoperated animals, aspirin decreased the respiratory rate, while in the operated ones, a slight increase was observed.

Sweating, normally present in animals subjected to a high body temperature in a hot room, or in fever, practically disappeared after the lesions.

Higher values in the plasma specific gravity were obtained after the operation, although blood dilution in response to heat and fever occurred. Plasma chloride values were also elevated after the operation; it is believed that both rises were due to loss of water from the blood.

Anterior and antero-lateral hypothalamic lesions suppressed the sweating mechanism of aspirin antipyresis in monkeys.

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INVESTIGATION OF THE ANTIBACTERIAL AND TOXIC ACTION OF CERTAIN ACRIDINE DERIVATIVES

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In 1910, Benda synthesised 2 8 diaminoacridine sulphate and 2 8 diamino 10 methyl acridinium chloride hydrochloride, and in the following year the latter was recommended by Ehrlich and Benda for use as a trypanocide, it was given the name trypaflavine   Shortly afterwards its excellent antiseptic properties were discovered by Browning, and it was used extensively in the treatment of wounds during the War of 1914–1918 under the name acriflavine   Although 2 8 diaminoacridine sulphate, or proflavine as it is now generally known, is also a good antiseptic, it never acquired the same popularity as acriflavine, possibly because, being much less soluble, it was less convenient to dispense

Originally, acriflavine was regarded as relatively non toxic with no appreciable inhibitory action on leucocytes and tissue regeneration when applied in bactericidal concentrations   It had the great advantage over the phenolic anti septics in use at the time of retaining its activity in presence of serum and pus Its main disadvantage was that it was relatively slow in action against many organisms   For many years acriflavine was widely used as a dressing for wounds and infective skin conditions and it was also used by the mouth and by irriga tion as a genito urinary antiseptic   It was also given intravenously in some of these conditions, apparently without ill effects   About 1932–1933, however, opinion concerning the value of acriflavine underwent a change, and occasional reports appeared of toxic manifestations, such as jaundice and liver damage, when the drug was given intravenously , it was also said to cause pain on injection and abscess formation   These toxic reactions appear to have been due mainly to a variation in the method of manufacture, and an investigation of the process led to the introduction in 1935 of a much less toxic form of acriflavine

Moreover, Gailhot (1) showed that acriflavine as ordinarily prepared is a mix ture of 2 8 diaminoacridine hydrochloride and 2 8 diamino 10 methyl ac ridinium chloride hydrochloride, it is therefore a substance of uncertain composition and consequently its properties may vary

The recognition that acriflavine sometimes produced serious reactions prompted Linnell and his colleagues to investigate other derivatives of acridine, and Albert and Linnell (2, 3, 4) described the preparation of several diamino acridines, the bacteriological properties of which were investigated by Albert, Dyer and Linnell (5), and by Albert, Francis, Garrod and Linnell (6)   The most interesting of the new compounds proved to be 2 7 diaminoacridine monohydro-

chloride.   Its relation to proflavine and acriflavine is illustrated by the following formulae:

2:7-diaminoacridine
monohydrochloride

2:8-diaminoacridine
sulphate (proflavine)

2:8-diamino-10-methyl-acridinium chloride hydrochloride

The new compound had similar bacteriostatic and bactericidal properties to proflavine, but was somewhat more toxic to leucocytes and more inhibitory to phagocytosis than proflavine.

Its systemic toxicity was, however, lower, the following values (mg./Kg.) being obtained for the L.D. 50:

2:7-Diaminoacridine monohydrochloride	500
Proflavine. . .	200
Acriflavine	50

Suffolk (7) found 2:7-diaminoacridine monohydrochloride to be less inhibitory to the acetyl choline effect in the isolated frog auricle than either acriflavine or proflavine, whilst Manifold (8, 9) observed a similar result in the effect of the compound on the oxygen uptake of brain tissue.   Russell and Falconer (10 and 11), using isotonic solutions buffered to pH 6.2, reported that 2:7-diaminoacridine monohydrochloride and proflavine, unlike the other compounds tested, did not cause haemorrhage or necrosis when applied to the exposed brain of rabbits.

The application of acridines to wounds has been criticised by several workers. Jacoby, Medawar and Willmer (12) found proflavine to be more toxic to fibroblasts, macrophages and epithelia than sulphonamides, whilst Abraham et al. (13) tested both proflavine and 2:7-diaminoacridine monohydrochloride for their action on leucocytes, and concluded that "the flavines are more toxic than was previously supposed, and are not suitable for repeated use in high concentration on such lesions as burns."

Garrod (14), however, pointed out that "if a wound can really be disinfected by something which also damages leucocytes, this is a small price to pay for such effect; leucocytes are easily and quickly replaced, and unless there is demon-

strable damage to fixed tissues as well, this degree of 'toxicity' is no bar to useful-ness in wounds involving connective tissue and muscle "

More recently, Albert, Rubbo and Goldacre (15), Albert, Goldacre and Rubbo (16), and Albert and Ritchie (17) prepared and tested a number of monoamino acridines, the best of which proved to be 5 aminoacridine hydrochloride   This compound was investigated more thoroughly by Rubbo, Albert and Maxwell (18), who showed that it was as active as proflavine against a number of organisms although its systemic toxicity was somewhat higher than that of proflavine It possessed the advantage of being non staining

2 7-Diaminoacridine monohydrochloride and 5 aminoacridine hydrochloride have now been tested *in vitro* and *in vivo* against a number of organisms, and their effects on body fluids and tissues have also been investigated   The tartrate of 2 7 diaminoacridine, prepared by Mr A E Bide and Mr R Peevers, has also been tested   In addition, following a suggestion made by Dr G Brownlee of the Wellcome Physiological Research Laboratories, 2 7 diaminoacridine base, 5 aminoacridine base and 2 8 diaminoacridine base were tested to see whether they were more innocuous to tissues than the corresponding salts

Bacteriostatic activity   The dilutions of acriflavine, proflavine, 2 7 diamino-acridine monohydrochloride and 5 aminoacridine hydrochloride required to inhibit the growth of various organisms, in digest broth, 10% serum broth and whole blood were estimated after 24 hours by the usual method   The results, shown graphically in figure 1, indicate that proflavine and, more so, acriflavine are more active against gram positive organisms than the 2 7 diamino and 5 amino compounds, whereas the last two proved to be more active than pro flavine against gram negative organisms, and only slightly inferior to acriflavine The results also indicate the difference between the effect of protein on acriflavine and on the other three compounds, for whereas in only one instance is the activity of acriflavine depressed by addition of blood, this is seldom the case with the activity of the other three compounds

The effect on the bacteriostatic activity of varying the pH of the medium in which the tests are carried out is illustrated in figure 2, which shows the bac teriostatic concentration of the four compounds against *Staphylococcus aureus* in digest broth at pH 6 6, 7 4, 7 8 and 8 6   The results indicate that the activity increases as the pH becomes more alkaline

Bactericidal activity   Figures 3, 4 and 5 indicate the rate at which *Staphylococ cus aureus*, *B coli*, *B proteus vulgaris* and *Ps pyocyanea* are killed by various concentrations of acriflavine, 2 7 diaminoacridine monohydrochloride and 5-aminoacridine hydrochloride   They show that acriflavine is very much more rapid in its action against *Staphylococcus*, but slower in its action on *B coli* and *B proteus* than 2 7 diaminoacridine hydrochloride, and that it is more rapid than 5 aminoacridine hydrochloride against these three organisms   Against *Ps pyocyanea* all these compounds have little bactericidal effect, though 2 7-diam-inoacridine hydrochloride and acriflavine are markedly bacteriostatic

The behaviour of 2 7 diaminoacridine monohydrochloride by Fleming's "gut-ter" method was compared with the behaviour of acriflavine   Two "gutters"

chloride.   Its relation to proflavine and acriflavine is illustrated by the following formulae:

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Proflavine 200
Acriflavine 50

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In vitro experiments showed that all three acridine salts and the corresponding bases were bactericidal against *Cl welchii, Cl oedematiens, Cl tetani* and *Cl novyi* in a medium containing meat at a dilution of 1 in 100,000   In glucose broth medium, the salts were bacteriostatic against these four organisms in dilutions up to 1 in 2,000,000 and the bases up to 1 in 500,000

Recently Withell (19) introduced a new expression to describe the bactericidal potency of a compound   This is the time necessary to reduce the number of bac
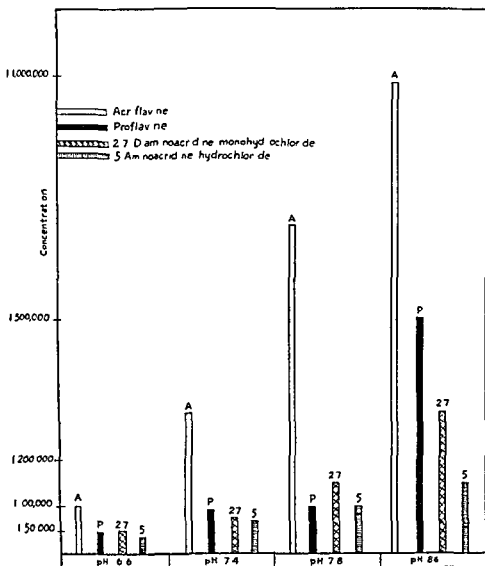
Fig 2

teria to 50% of the original count, and is designated L T 50   According to Hobbs and Wilson (20), the method has advantages over the measurement of the reaction velocity constant (K) in the middle phase of growth   The procedure as described by Withell was applied to the acridines except that the substances were tested in serum instead of water or saline   There were two reasons for using serum, first, the acridine bases are sufficiently soluble in serum, though not in water, to give the necessary dilutions, and secondly, the organisms retain their

viability better in serum than in water or saline over a period up to 3–4 hours. During this time the effects of multiplication and inhibition by the serum are negligible.   Ten-ml. quantities of sterile horse serum were introduced into sterile pyrex boiling tubes, and solutions of the compounds in dilutions of 1 in 5,000 and

Staphylococcus
B. coli
B. proteus
Ps. pyocyanea
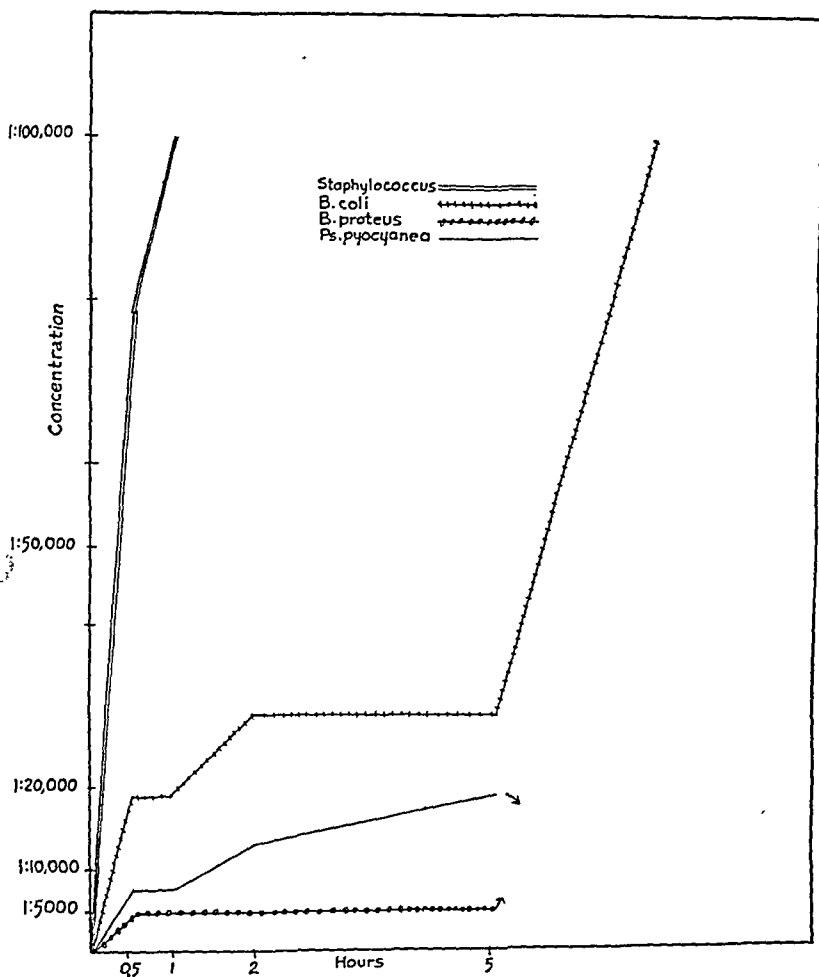
Fig. 3

1 in 10,000 were added; a similar tube containing no acridine was used as control. Fresh 18-hours old agar cultures of *Staphylococcus aureus* (strain 663) and *B. coli* (strain 613) were suspended in saline and 0.1 ml. of the suspension was added to each tube so that the final concentration of *Staphylococcus* was 10,000 organisms

per ml. and of *B. coli* 5,000 per ml. The tubes were immersed in a water-bath at 37°C.; 3 drops were removed at intervals by means of standardised Pasteur pipettes, transferred to tubes of melted digest agar at 48°C. and the whole poured into Petri dishes. The amount of acridine present in the 3 drops of inoculum was
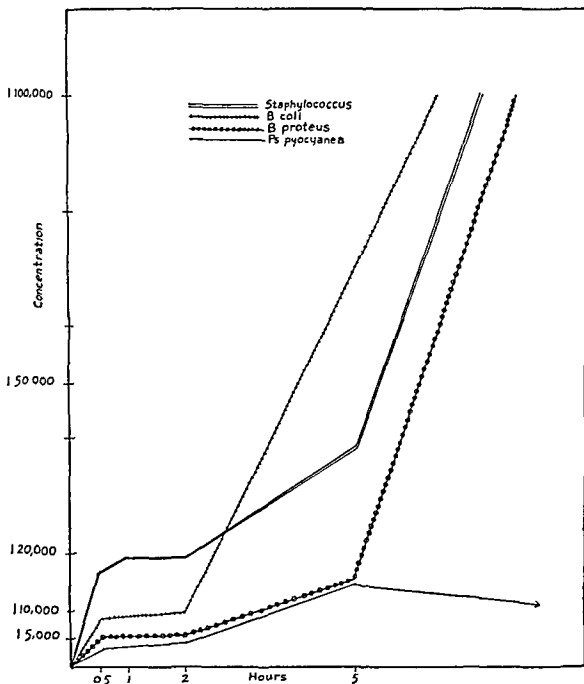
FIG. 4

so small that it could not interfere with the growth of the colonies. The results, which are the average of repeated experiments, are given in table 1, the figures indicating the value of L.T. 50 in minutes.

These results indicate that in serum 2:7-diaminoacridine hydrochloride kills

Staphylococcus aureus more quickly than the other compounds, and that in general the salts kill more quickly than the bases. The observation that 2:7-diaminoacridine tartrate is much slower in its action than the hydrochloride was quite unexpected and, at the moment, inexplicable.
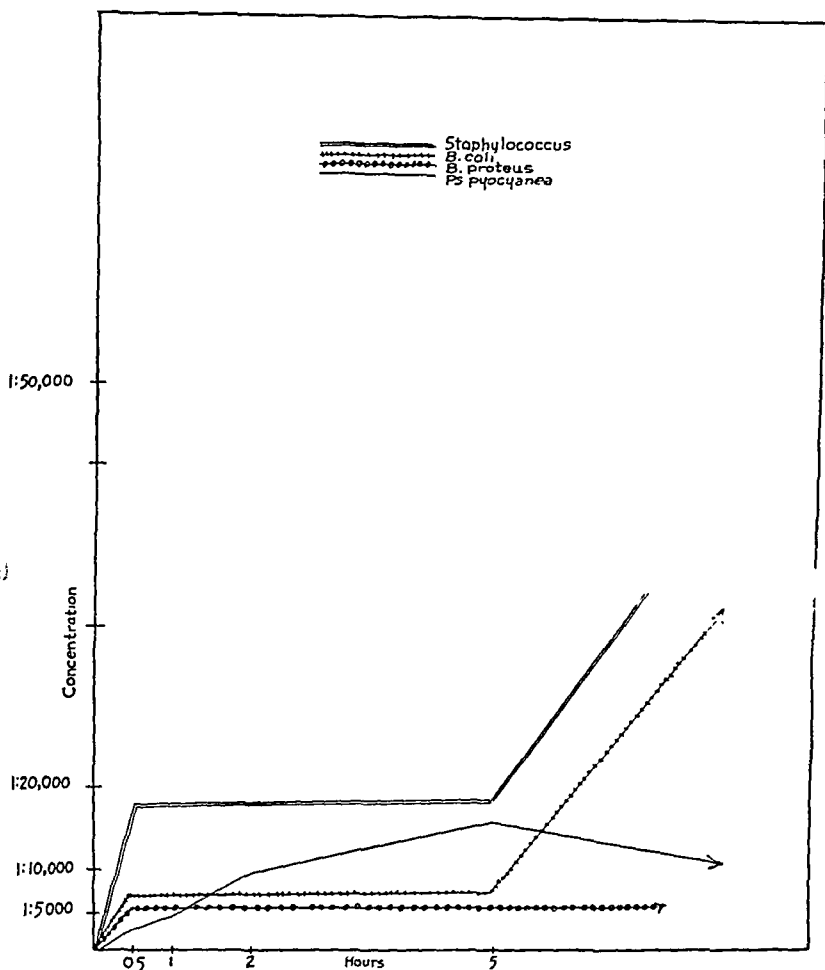
FIG. 5

Trypanocidal activity. 2:7-Diaminoacridine hydrochloride had no effect on *Trypanosoma equiperdum*, but had some effect against *T. gambiense*; after intravenous or intraperitoneal injection about 70% of a group of mice were cured by 0.1-ml. doses of a 0.1% aqueous solution. This dosage also protected mice infected with *Spirochaeta duttoni* when given daily for 6–12 days.

Effect on toxins. Zuntz states that acridine derivatives neutralise bacterial toxins Experiments on staphylococcus and diphtheria toxins, however, showed that 5-aminoacridine hydrochloride, 2:7-diaminoacridine monohydrochloride and acriflavine had no effect on staphylococcus haemolysin when tested on rabbits' red cells in dilutions of 1 in 500 to 1 in 10,000, and that diphtheria toxin, tested on guinea pig's skin, was not neutralised by dilutions of 1 in 500 to 1 in 50,000

Toxicity. The value of L.D. 50 was determined by Mr. M. R. A. Chance and Mr. T. R. Middleton, the solutions being adjusted to a pH of about 6 0 by addition of phosphate buffer solution   Doses were given intraperitoneally at such
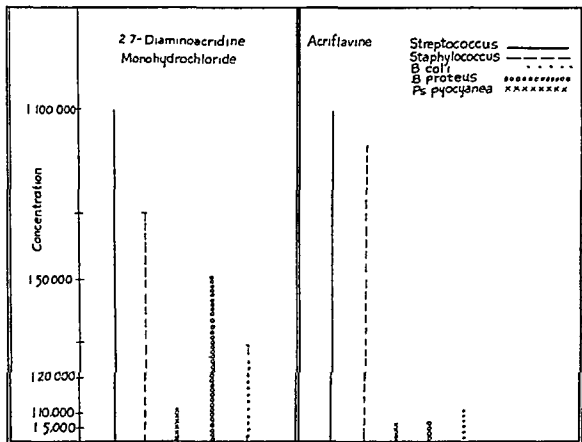
Fig 6

concentrations that the amount administered per 10-g. body weight was present in 0 5 ml.   The following values were obtained:

Acriflavine	65
2 7 Diaminoacridine monohydrochloride	300
5-Aminoacridine hydrochloride	70 mg per kg.

These results are in agreement with those previously reported. The relatively high toxicity of acriflavine is not, therefore, due to the low pH of its solution since a similar value for L.D. 50 was obtained with an unbuffered solution.

Effect in blood and plasma   The concentrations of the compounds which just

gave no visible precipitate with plasma, and the concentrations which just failed to produce haemolysis of a 2% suspension of fresh rabbit red cells were measured and are recorded (in mg. per 100 ml.) in table 2, together with the solubility of the compounds in saline and the pH of the resulting solutions.

Tissue culture experiments. Tests were carried out by the slide technique on chicken embryo heart. Graded dilutions of the acridine derivatives were added to the nutrient plasma, and the rate of growth was noted after 24, 48 and 72 hours. The results, which are the average of repeated experiments, were obtained as given in table 3.

Under the same conditions, sulphapyridine inhibited the growth of fibroblasts at 1 in 500, but not at 1 in 1000. It does not follow, however, that the acridines are therefore too toxic to be administered locally, as the mechanism of the action of the two groups of compounds is quite different; the acridines are bactericidal, and require only one or two applications, whereas the sulphonamides, being bacteriostatic, must be applied over a longer period. It is not yet clear to what

TABLE 1

SUBSTANCE	STAPH. AUREUS			B. COLI		
	Temp. 37°C.		Temp. 21°	Temp. 37°		Temp. 21°
	1:5000	1:10,000	1:5000	1:5000	1:10,000	1:5000
2:7-base..........................	85	150	>180	130	>180	>180
2:7-hydrochloride...................	9	9	35	4	20	15
2:7-tartrate........................	75	80		10	40	
5-base.............................	75	105		4	35	15
5-hydrochloride.....................	25	40	35	6	8	25
2:8-base...........................	55	105	100	12	12	15
2:8-sulphate.......................	55	95	100	4	12	20

extent observations on the growth of fibroblasts can be used for predicting possible damage to the tissue, and for this purpose experiments on artificial wounds are probably more suitable.

Tests on leucocytes. Leucocytes still retained their motility after 1 hour at 37°C. in 1 in 10,000 solutions of 5-aminoacridine base and hydrochloride, 2:7-diaminoacridine base, hydrochloride and tartrate and 2:8-diaminoacridine base and sulphate. They were immobilised by dilutions of 1 in 5000.

The effect of acridines on the phagocytic activity of leucocytes was tested, using a killed suspension of *Staphylococcus aureus*. Graded dilutions of each of the acridine compounds were added to an isotonic salt solution containing a leucocyte suspension from peritoneal exudate obtained by injecting guinea-pigs with a 0.1% starch-broth. The mixture was incubated for 1 hour at 37°. It was observed that a 1 in 10,000 solution of proflavine reduced phagocytosis about 24%, whilst the 2:7-diamino and 5-amino compounds in these dilutions were without effect.

Proflavine in a dilution of 1 in 5,000 inhibited phagocytosis still more, whilst

the 2:7-diamino and 5-amino compounds in this dilution reduced the activity about 50%.

Animal experiments. The effects of the acridine compounds on tissue were compared by injecting 0.2 ml. of a 0.5% suspension of each into both flanks of four rabbits subcutaneously. 5-Amino-acridine base and hydrochloride caused only local irritation limited to the area of injection; this disappeared in 2–3 days, leaving a transitory discolouration of the skin. 2:7-Diaminoacridine base formed a depot in the subcutaneous tissue, due to the insolubility of the substance and within a few days this changed into a hard nodule, persisting over a period

TABLE 2

	SOLUBILITY IN SALINE	pH AT 20°C.	PLASMA REACTION AT 20°C.	HAEMOLYTIC EFFECT AT 20°C.
2:7-base..	0.36		0.06	
2:7-HCl.........	400	5.8	26	50
2:7-tartrate............	1,200		1.6	40
2:8-base....	0.55		0.1	0.38
2:8-sulphate..	300	2.2	30	100
5-base	0.47		0.25	4.7
5-HCl...........	142	6.0	9	30
Acriflavine..	200	2.0	10	30

TABLE 3

	DILUTION	
	1 in 100,000	1 in 200,000
Acriflavine...............	0*	+
Proflavine.	±	++
2:7-Hydrochloride..................	+	+++
5-Hydrochloride	±	++
	1 in 100,000	1 in 30,000
2:8, 2:7 and 5-bases....	+	0

* Few fibroblasts noticeable after 24 hours.

0, no growth; ±, weak growth; +, slight growth; ++, normal growth; +++, good growth.

of 14–21 days; in some animals the skin over the nodule became necrotic. 2:7-Diaminoacridine hydrochloride and tartrate produced a transitory pinkish discolouration of the skin, followed by the formation over the injected area of a scab, which separated in the course of a week. 2:8-Diaminoacridine base produced a skin lesion resembling that produced by the 2:7-base, but the rate of absorption was much quicker; the scab resembled that caused by 2:8-diaminoacridine sulphate (proflavine). Proflavine produced more damage to the subcutaneous tissue than did any of the other compounds; the skin over the injected area became necrotic in 3–4 days, and a craterlike ulcer covered with a firmly

adherent scab persisted for 10–14 days. In an endeavour to explain the more extensive damage caused by proflavine solutions, the pH of which is about 2.0, the effect of hydrochloric acid solutions of pH 2, 4 and 6 on subcutaneous tissue, was tested. The amounts used (0.2 ml.) did not produce any noticeable damage to the tissue, however, and it would there appear that the damaging effect is not due to the low pH of proflavine solutions. This was confirmed by the fact that injections of a 0.5% solution of proflavine buffered to pH 7.0 also produced lesions in the subcutaneous tissue similar to those produced by unbuffered solutions.

Effect on wound healing. The skin on the flanks of 6 rabbits was shaved, and under general anaesthesia excisions extending into the corium were made; these were about $\frac{1}{2}''$ in diameter. The wounds were dressed with gauze soaked in a 0.1% solution of 2:7-diaminoacridine monohydrochloride twice daily for 5 days. The process of healing went on satisfactorily, granulation tissue was well vascularised at the base of the wound in the first few days, and epidermis restitution under the crust continued undisturbed.

In another series of experiments groups of 3 rabbits and 3 guinea-pigs under a general anaesthetic were cauterized on the shaved skin of the back with an electric iron to produce wounds equal in size. The scab which formed was removed after 5 days, revealing a wound of approximately 2 cm. square. The exposed wound on one flank was then dressed on alternate days with the powdered acridine and covered with lint until the control wound on the other flank (which was dressed without any antiseptic) had healed; this was approximately 20 days. Measurements of the area were made every four days. The application of powdered proflavine, acriflavine and 5-amino-acridine hydrochloride to the wounds produced 5 days previously and from which the scab had been removed prevented healing, proflavine being the most and 5-amino-acridine hydrochloride the least inhibitory. Acriflavine, and to an even greater extent proflavine and the corresponding base, produced at the base of the wound a thick, firmly adherent scab which was difficult to remove. The presence of the powder on the treated wound apparently did not interfere with the healing of the control lesion on the opposite flank. 2:7-Diaminoacridine monohydrochloride produced only a thin, pliable scab, which separated easily from the wound, and the rate of healing was only slightly less than that of the controls. The corresponding base was even less inhibitory to wound healing, the extent of epithelization being almost identical with that of the control wounds. 5-Aminoacridine hydrochloride and the corresponding base had a delaying effect on wound healing, but the scab came off readily, leaving the ulcer bleeding freely. We noticed that the rate of healing of the control untreated wounds was less than in other groups of guinea pigs and the amount of bleeding was more extensive. It has yet to be shown if this is a systemic effect due to absorption of the compound.

The effect of the compounds on infected wounds was also tested. Artificial wounds in rabbits were infected with fresh suspensions of *Streptococcus haemolyticus* and *Ps. pyocyanea*. The next day and daily thereafter the wounds were treated with powdered 2:7-diaminoacridine hydrochloride and acriflavine, and

the wounds were examined daily for bacteria   The rate of healing was greater, and the granulation tissue looked healthier, with the 2 7- compound than with acriflavine, and pus formation continued for a longer period of time with ac riflavine

Other experiments were made on rabbits which, after laparotomy, had devel oped fistulae from which *Staphylococcus aureus* and *Ps pyocyanca* were isolated Treatment with acriflavine had no effect, but as soon as dressings of 2 7 diamino acridine hydrochloride were applied, the pus diminished and granulation tissue appeared   The *Staphylococcus* infection cleared up in 2 days, and only *Ps pyocyanea* was recovered from the nearly closed wounds

Pharmacological properties   A preliminary investigation of the action of 2 7-diaminoacridine hydrochloride, 5 aminoacridine hydrochloride, proflavine and acriflavine on plain muscle was made by Mr M R A Chance and Mr T R Middleton, using isolated strips of ileum and uterus in oxygenated Ringer's solution   They have also observed the effect of these substances in the blood vessels of the ear of amytal anaesthetised rabbits by intravenous injection All three substances caused contractions of plain muscle in dilutions from 1 in 10,000,000 to 1 in 100,000

The hydrochlorides of 5 amino acridine and 2 7 diaminoacridine had a greater effect and acted in lower concentrations than either proflavine or acriflavine In addition the 2 7 and 5 amino compounds increased muscle tone

DISCUSSION   Antiseptics should be chemically stable, and there should be as wide a difference as possible between the therapeutic and toxic doses   They may be bacteriostatic or bactericidal, depending on such factors as the con centration of the solution used, temperature, time of action, pH of the medium, presence of protein in the medium, and the rate of absorption and excretion when administered to patients   It is obviously an advantage for the com pound to be of a non sensitizing character

The purpose of an investigation such as that now described is to determine the extent to which a new compound is likely to be satisfactory when used clin ically, the conditions in which it is most likely to be successful, and the nature of the risks to which patients treated with a new and untried drug are exposed

The method of carrying out *in vitro* tests has undergone a change in recent years   The once widely used Rideal Walker test is now held to have a very limited sphere of usefulness, and more refined techniques, such as those of Watson, Phelps, Chick and Withell, are preferred   Unfortunately, these meth ods often give different results in the hands of different workers, comparative tests, however, carried out in the same laboratory are valuable in helping to select a substance for a specific purpose

An antiseptic which is to be administered locally or parenterally is subject to a number of factors in addition to those mentioned above   It is, for example, liable to be inactivated by enzymes or converted to inert substances by other mechanisms of the body   Generally also it has to cope with different infecting organisms, and it is difficult to predict to what extent it will be effective in all these cases   The degree of interference with fibroblast production and leuco

cyte activity should also be determined as giving an indication of its possible action in the body, and, finally, the action of the drug on various tissues of the body must be considered, and the extent of the irritation provoked on injection must be determined. Often information about such reactions is of greater importance than a knowledge of the actual value of L.D. 50 in animals, although this is obviously essential when the drug circulates in the body.

The results recorded in this paper indicate that both 5-amino-acridine hydrochloride and 2:7-diaminoacridine monohydrochloride are potent antiseptics, and possess certain advantages over acriflavine and proflavine. The *in vitro* experiments show that the 2:7-diamino-compound will be of advantage where a rapid action is required, whilst its relatively high solubility and rate of absorption, together with its low systemic toxicity, suggest that it may be of use as an oral or systemic antiseptic. Preliminary experiments have shown that it is excreted partly unchanged in the urine, and if the concentration is shown to be sufficiently high, the substance may be of use as a urinary antiseptic. Both 2:7-diaminoacridine monohydrochloride and 5-amino-acridine hydrochloride are less toxic to tissue than proflavine or acriflavine, and do not interfere with the healing of artificial wounds to the same extent. For this reason, both are preferable to the older antiseptics for routine use, in spite of the fact that they have a lower bactericidal activity than acriflavine. 5-Aminoacridine hydrochloride has the added advantage of being non-staining.

Note on chemical and physical properties. 2:7-Diaminoacridine monohydrochloride, $C_{13}H_{11}N_3 \cdot HCl$, forms almost black crystals with a greenish lustre; these decompose at 365–370°C. without melting. The compound was prepared by a new method recently discovered by Albert (21).

It has a characteristic absorption spectrum with maxima at 365, 284, and 235 $m\mu$ of which the 284 $m\mu$ band is the most prominent; at this wave-length $E_{1cm}^{1\%}$ is 2840 calculated as the free base. The value of the extinction coefficient is constant between pH 6.0 and pH 3.5 but is lower, with the maximum slightly displaced, at lower pH values. In 50% aqueous alcoholic N/100 sodium hydroxide solution the most prominent absorption band is at 273 $m\mu$ and the extinction coefficient is 4600 calculated as the free base. The free base is a golden-yellow powder which decomposes at 355°C. and is much less soluble than the hydrochloride. Both the base and the hydrochloride give strongly fluorescent solutions in alcohol and other organic solvents; the aqueous solutions are only slightly fluorescent.

2:7-Diaminoacridine monohydrochloride has a solubility of 3.3% in water, and the pH of its aqueous solutions (0.25 to 3.0%) is 5.6.

An isotonic 0.1% solution of pH 6.3 can be made by dissolving 0.1 g. of 2:7-diaminoacridine monohydrochloride, 0.2 g. of $NaH_2PO_4 \cdot 2H_2O$, 0.2 g. of $Na_2HPO_4 \cdot 12 H_2O$ and 0.8 g. of sodium chloride in water and diluting to 100 ml.

Solutions stain the skin and fabric pink or red. The colour can be removed from cloth by washing in warm water and dilute bleaching powder solution and then rinsing with water.

Aqueous solutions are not very stable to light and should be stored in amber bottles.

2 7-Diaminoacridine tartrate, $(C_{13}H_{11}N_3)_2 \cdot C_4H_6O_6$, is a maroon coloured powder, which decomposes on heating   It is extremely soluble in water and the pH of a 1% solution is 5 6   5 Aminoacridine hydrochloride, $C_{13}H_{10}N_2 \cdot HCl$, is a yellow crystalline substance which does not melt at 355°C   It was prepared by the method of Albert and Ritchie (22, 23)   It is soluble in water to the extent of 1 2%, giving a pale yellow solution, pH 6 6, its solubility in half isotonic saline is 0 1%

An isotonic 0 1% solution of pH 6 8 can be made by dissolving 0 1 g of 5 amino acridine hydrochloride, 0 05 g of borax, 1 2 g of boric acid and 0 3 g of sodium chloride in water and diluting to 100 ml

The solutions, unlike those of the other acridines, do not stain skin or fabric   A 0 1% solution is yellow with a faint green fluorescence, whilst a 0 001% solution is colourless, with an intense blue fluorescence   In 50% aqueous alcoholic N/100 sodium hydroxide solution, it has an absorption spectrum with a maximum at 262 mμ, at which wave length $E_{1cm}^{1\%}$ is 3640 calculated as the free base

Solutions are more stable to heat and light than are solutions of other acridine antiseptics, but they should, nevertheless, be preserved in amber bottles   The free base is a yellow crystalline substance, m p 234–235°C , which is less soluble in water than the hydrochloride

SUMMARY

1   2 7 Diaminoacridine monohydrochloride and 5 aminoacridine hydrochloride are active in high dilutions against gram positive cocci (streptococcus, pneumococcus, staphylococcus) and spore bearing anaerobes and in lower dilutions against gram positive and gram negative bacilli   The presence of blood and serum decreases the activity of both compounds to some extent   They do not neutralise toxin *in vitro*

2   Whilst acriflavine is more rapid than 2 7 diaminoacridine monohydrochloride in its bactericidal effect against staphylococcus, it is slower against *B coli* and *B proteus*   5 Aminoacridine hydrochloride is the least rapid of the three compounds

3   In serum, 2 7 diaminoacridine monohydrochloride kills staphylococcus more quickly than do 5 aminoacridine hydrochloride and proflavine, the three compounds are approximately equally effective against *B coli*   The salts are in general more rapid in their action than the corresponding bases

4   2 7 Diaminoacridine monohydrochloride has a lower, and 5 aminoacridine hydrochloride a higher, systemic toxicity than proflavine, but both compounds are less toxic to tissues than proflavine and acriflavine   The action of the bases on tissues was similar to that of the corresponding salts

5   The 2 7 compound interfered least with the healing of artificial wounds, whilst the 5 amino compound was also less harmful than proflavine or acriflavine   The order in which the compounds slowed down the rate of healing was the same whether the substances were applied in solution or in powder form

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PHENOL STUDIES VI

The Acute and Comparative Toxicity of Phenol and o-, m- and p-Cresols
for Experimental Animals

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Phenol and cresol are manufactured in large quantities for the preparation of
a great variety of substances, and their handling gives rise to some hazard from
regularly or frequently repeated exposures, as well as to serious danger from ex-
tensive accidental contact. For this reason the toxicity of these compounds
has been made the subject of further investigation. Signs of acute poisoning
induced by the absorption of phenol and the three cresols are very similar.
They have been described accurately by earlier investigators (1, 2, 3, 4, 5)
whose observations have been well summarized by Ellinger (6).

EXPERIMENTAL METHODS. Rats, rabbits and cats were employed as experimental ani-
mals. The rats, Wistar Institute stock, were reared in the laboratory; unless otherwise
specified, they weighed from 100 to 200 gm. Albino rabbits ranging in weight from 2 to 3
kg. were purchased from a local breeder and kept under observation for about two weeks
before being used. The cats were stray animals, kept under observation in isolation for
one month to establish their state of health. Equal numbers of males and females were
employed in individual experiments.

A rubber catheter passed through the esophagus was usually used for the oral adminis-
tration of materials to rabbits. In a few instances, however, the amounts to be adminis-
tered were 1 ml. or less, and since it was desirable to avoid dilution of the compound, by
rinsing of the catheter, the substance was injected directly through the abdominal wall
into the stomach by means of a syringe and hypodermic needle. A blunt hypodermic needle
traversing the esophagus, was employed for like purpose in the case of rats. Intravenous
injections were given at a rate of 1 ml./minute into the marginal ear vein of rabbits. In
preparation for skin applications, in the case of the rabbits, the hair was clipped very short
on the belly or the back over an area of about 20 square inches, unless otherwise indicated;
in the case of rats, the materials were applied without any preliminary attempt to remove
the hair. All animals were supplied with food and water up to the time of treatment.

The phenol used was Merck's reagent quality, and the o-, m- and p-cresols, obtained
from the Eastman Kodak Co., were from 96 to 98 per cent pure. The melting points of these
cresols are 30–31°, 10–11°, and 32–34°, respectively. Unless stated otherwise, phenol was
administered as 2 and 5 per cent aqueous solutions, as emulsions in water containing 10,
20 and 50 gm. of phenol per 100 gm. of emulsion, and as solutions of water in phenol contain-
ing 75 or 90 gm. of phenol per 100 gm. of solution.

The relative toxicity of phenol and the three cresols was determined under conditions
that were kept identical for all four compounds; that is, in each experiment the same species
of animals, the same mode of administration, and corresponding concentrations and sol-
vents, were employed.

SIGNS OF POISONING. The first twitching noted in rabbits, rats, and cats to
which phenol had been administered occurred almost invariably in the extrinsic
eye muscles and those of the eyelids and ears, then spread to isolated bundles of

muscles all over the body, the extremities being the last to be affected. The body temperatures of all animals fluctuated, but for the most part were subnormal. Pulse and respiration were increased in rate at first, but later became slow, irregular and weak. The pupils were contracted in the early stages of intoxication, being dilated later. There was some salivation, and dyspnea was marked. Rats usually showed twitching of isolated bundles of muscles and uncoördinated movements of the legs until shortly before death. In the case of cats, the convulsions were marked but they gradually diminished, giving way to a state of lethargy and coma. (While in coma the cats could be thrown back into convulsions by auditory stimuli.) Rabbits behaved similarly but showed in addition asphyxial convulsions just before death. Most of the animals died in from 15 minutes to $2\frac{1}{2}$ hours, but there were a few late deaths (2 to 7 days) among rats and rabbits subjected to cutaneous applications, and also among cats injected subcutaneously. The survival time, in almost all cases, varied inversely with the size of the dose administered.

Signs and symptoms of poisoning produced by the three cresols resembled those caused by phenol, but on the whole convulsions were less severe, while the signs of weakness and collapse, and the depth of coma were more striking.

ORAL ADMINISTRATION OF PHENOL. Rabbits were given single doses (0.28, 0.42, 0.62 and 0.94 gm./kg.) of melted crystals of phenol or of aqueous solutions and emulsions prepared so as to contain 2, 5, 10, 20, 50, 75 and 90 per cent of phenol. (The reason for choosing these doses is described elsewhere (7).) The results, summarized in table 1, show that there is very little difference in the toxicity of dilute and concentrated preparations of phenol when administered orally. Lethal effects were produced uniformly by the dose of 0.62 gm./kg., sometimes by the dose of 0.42 gm./kg., but never by the dose of 0.28 gm./kg.

Table 2 summarizes corresponding data for rats, but the preparations administered were limited to those containing 2, 5, 10 and 20 per cent of phenol. The first three of these preparations showed the same degree of toxicity, the lethal doses (LD$_{50}$) being 0.53, 0.53 and 0.54 gm./kg., respectively. The 20 per cent emulsion was somewhat more toxic, the corresponding lethal dose (LD$_{50}$) being 0.34 gm./kg.

CUTANEOUS APPLICATION OF PHENOL. Preliminary studies had made it appear that melted phenol crystals or concentrated preparations of phenol, when applied cutaneously, were much less likely to produce systemic poisoning and death than dilute preparations. (Some of these data are shown in table 3.) The following experiment, however, demonstrated that this is not the case. It proved that the concentration of the phenol does not play the major rôle.

An area, $1\frac{1}{2}$ by 3 inches, on the abdominal skin of each of a series of rabbits was exposed for one hour to a solution containing 7, 75 or 95 per cent of phenol, the solution being confined within a chamber attached to the skin by latex. The extent of the absorption of phenol was determined by estimating the total phenol in the blood taken from these rabbits by heart puncture at the end of the hour. (Determinations were made by a spectrophotometric method described previously (8).) The values found in the case of seven animals exposed to the 7 per

cent solution were 1 1, 2 2, 2 4, 2 7, 3 9, 4 4 and 5 2 mg per 100 ml of blood, in the case of seven animals exposed to the 75 per cent solution of phenol they were 1 2, 2 5, 3 0, 3 8, 3 9, 4 7 and 5 1 mg per 100 ml, while in the case of eight exposed to 95 per cent phenol they were 2 2, 2 3, 3 2, 3 3, 4 0, 4 3, 4 8 and 6 0 mg per 100 ml   These data justify the conclusion that the extent of the absorption

TABLE 1

Comparative toxicity of aqueous preparations of phenol in different concentrations administered orally to rabbits

	NO OF RABBITS	DOSE	NO OF DEATHS	TIME TILL DEATH
		gm /kg		*min*
Solution 2 gm phenol and 98 gm water	1	0 42		survived
	2	0 62	1	30
	1	0 94	1	18
Solution 5 gm phenol and 95 gm water	1	0 28		survived
	1	0 42	1	00
	10	0 62	9	30–110
Emulsion 10 gm phenol and 90 gm water	2	0 42		survived
	2	0 62	2	40 and 60
	1	0 94	1	25
Emulsion 20 gm phenol and 80 gm water	1	0 42		survived
	1	0 62	1	30
	1	0 94	1	45
Emulsion 50 gm phenol and 50 gm water	1	0 28		survived
	1	0 42	1	40
	2	0 62	2	30 and 40
Solution 75 gm phenol and 25 gm water	1	0 42		survived
	1	0 62	1	20
	1	0 94	1	24
Solution 90 gm phenol and 10 gm water	1	0 28		survived
	2	0 42	2	18 and 36
	1	0 62	1	30
Phenol reagent Merck, heated to 40°C	1	0 28		survived
	1	0 42	1	16
	1	0 62	1	16

of phenol by a unit area of skin is little influenced by the concentration of the solution applied, within the limits investigated, but is largely dependent upon the magnitude of the skin area exposed

SUBCUTANEOUS, INTRAPERITONEAL AND INTRAVENOUS INJECTION OF PHENOL The dose of phenol that will kill approximately 50 per cent of rabbits given subcutaneous or intraperitoneal injections of a 5 per cent aqueous solution is 0 62

TABLE 2

Comparative toxicity of aqueous preparations of phenol in different concentrations administered orally to rats

NUMBER OF RATS USED	DOSE	PER CENT OF DEATHS	TIME TILL DEATH
	2% aqueous solution		
	$LD_{50} = 0.53$ gm./kg.		
	gm./kg.		*min.*
5	0.4	20	25
10	0.5	40	15–150
10	0.6	70	19–50
10	0.7	80	14–60
10	0.8	100	10–90
	5% aqueous solution		
	$LD_{50} = 0.53$ gm./kg.		
15	0.4	7	20
15	0.5	40	10–30
15	0.6	73	3–80
10	0.7	90	4–50
	10% aqueous emulsion		
	$LD_{50} = 0.54$ gm./kg.		
10	0.5	40	15–35
10	0.6	60	10–50
10	0.7	90	10–120
10	0.8	90	7–60
	20% aqueous emulsion		
	$LD_{50} = 0.34$ gm./kg.		
15	0.3	40	5–45
15	0.4	60	15–60
15	0.5	100	5–55

TABLE 3

Comparative toxicity of aqueous preparations of phenol in different concentrations applied to the abdominal skin of rabbits

(Standard dose for all concentrations = 2 gm. phenol per kilogram of rabbit)

DOSE 2 GM/KG, ADMINISTERED AS	NUMBER OF RABBITS USED	PER CENT OF DEATHS
Emulsion: 10 gm. phenol and 90 gm. water	10	100
Emulsion: 25 gm. phenol and 75 gm. water	10	90
Emulsion: 50 gm. phenol and 50 gm. water	10	90
Solution: 75 gm. phenol and 25 gm. water	10	80
Solution: 90 gm. phenol and 10 gm. water	10	50
Solution: 95 gm. phenol and 5 gm. water	17	53
Melted phenol reagent heated to 40°C.	15	30

gm /kg , the corresponding lethal dose by intravenous administration is about 0 18 gm /kg . The lethal dose for cats when injected subcutaneously as a 10 per cent solution in olive oil, is approximately 0 08 gm /kg

EFFECT OF PHENOL ON RATS OF DIFFERENT AGES   Two groups of young rats, the first 10 days old, the second 5 weeks, were selected, while a third group was composed of adult animals   Half the rats in each of these groups were subjected to skin applications of phenol each rat receiving one application of 3 gm /kg , each of the others was given 0 6 gm /kg orally   The results of these experiments, shown in table 4, demonstrate that susceptibility to phenol poisoning was greatest in the 10 day old rats, less in the adult rats, and least in the 5 week old animals

THE COMPARATIVE TOXICITY OF PHENOL AND o , m AND p CRESOL   Four groups of animals were used to determine the relative toxicity of phenol and the 3 cresols   Each of the 4 compounds was injected subcutaneously, as a 10 per cent solution in olive oil, into cats (table 5)   o Cresol was the most toxic, phenol

TABLE 4

Difference in susceptibility of rats of varying ages to phenol (5% aqueous solution) administered orally and cutaneously

	NUMBER OF RATS USED	DOSE	PER CENT DEAD	TIME TILL DEATH
		gm /kg		
Adult rats	20	3 0 cutaneously	45	30–180 min
	20	0 6 orally	60	30– 65 min
5-week old rats	20	3 0 cutaneously	25	2– 3 hours
	30	0 6 orally	30	30– 90 min
10 day old rats	20	3 0 cutaneously	65	2– 14 hours
	20	0 6 orally	90	12 24 hours

and p cresol came next and were of equal toxicity, while m cresol was the least toxic   When given orally to rabbits in the form of 20 per cent aqueous emulsions (table 6), phenol was the most toxic, p and o cresol were next and were practically on a par, while m cresol was again the least toxic   When injected into rabbits intravenously (0 5 per cent aqueous solutions), phenol, o and p cresol showed the same degree of toxicity, m cresol being again the least toxic (table 7)   When administered orally to rats in the form of 10 per cent solutions in olive oil, o cresol was the most toxic, and was followed by phenol p cresol, and finally m cresol (table 8)

COMPARISON OF METHODS FOR REMOVING PHENOL FROM THE SKIN   Alcohol has been the time honored solvent for removing phenol accidentally spilled upon the skin   Since the removal of phenol from large areas of the body would necessitate the use of large amounts of alcohol, and since previous studies carried out in this laboratory had shown that alcohol is absorbed through the skin in sufficient quantities to produce poisoning the possibility of employing soap and water as a substitute was investigated

Phenol (3 gm./kg.) was applied in the form of a 6 per cent aqueous solution upon the skin of each of a series of rats. This dose covered about one-sixth of the total body surface. The animals were returned to their cages and after varying periods of time were washed with 30 ml. of alcohol or with soap and water (using cotton swabs). Of 15 rats washed in 15 minutes with a 50 per cent solution of alcohol in water, 12 survived, but of 15 rats washed in this manner

TABLE 5

Single doses of phenol and cresols (10% in olive oil) lethal to cats, by subcutaneous administration

(One animal was used in each instance)

DOSE	PHENOL	o-CRESOL	m-CRESOL	p-CRESOL
gm./kg.	*hours till death*			
0.024		survived		
0.036	survived	survived		survived
0.055	survived	60		survived
0.080	312	60	survived	120
0.12	10	22	survived	84
0.18	7	8.5	27	21
0.28	2	3	4	4.5
0.42	6		12	10
0.62	8	7	7	8
0.94	6	4	5.5	7

TABLE 6

Single doses of phenol and cresols (20% aqueous emulsions) lethal to rabbits, by oral administration

(One animal was used in each instance)

DOSE	PHENOL	o-CRESOL	m-CRESOL	p-CRESOL
gm./kg.	*time till death*			
0.18	survived			
0.28	survived			survived
0.42	30 min.	survived		survived
0.62	45 min.	survived	survived	4 hrs.
0.94	30 min.	4 hrs.	survived	12 hrs.
1.40		40 min.	8 hrs.	2 hrs.
2.10			90 min.	

after 30 minutes had elapsed, only 6 survived. When 25 per cent alcohol was employed for cleaning two corresponding groups of animals, the numbers of surviving animals were 9 and 4, respectively, but when 95 per cent alcohol was similarly used, the numbers of survivors dropped to 6 and 3, respectively. When soap and water was employed to remove the phenol and the animals were washed in 15 minutes and in 30 minutes, respectively, the survivors numbered 12 and 6,

respectively Twenty two rats were subjected to a corresponding application of phenol, without benefit of any attempts at the removal of the material All these animals died promptly

TABLE 7

Single doses of phenol and cresols (0 5% aqueous solutions) lethal to rabbits by intravenous injection

(One animal was used in each instance)

DOSE	PHENOL	o-CRESOL	m CRESOL	p-CRESOL
gm /kg		*hours t ll death*		
0 08	survived	survived		survived
0 12	survived	survived	survived	survived
0 18	3	8	survived	15
0 28	2 5	10	15	10
0 42			7	

TABLE 8

Single doses of phenol and cresols (10% solution in olive oil) lethal to rats by oral administration

(10 rats were used in each instance)

DOSE	PHENOL (LD₅₀ = 1 5 GM /KG)	o-CRESOL (LD₅₀ = 1 35 GM /KG)	m-CRESOL (LD₅₀ = 2 01 GM /KG)	p-CRESOL (LD₅₀ = 1 8 GM /KG)
gm /kg		*per cent dead*		
1 0	0	20		
1 1	10			
1 2	30	50		
1 3	53			20
1 4		40		
1 5	53		0	40
1 6		60		
1 7			20	
1 8	60	90		30
2 0	80	100	40	50
2 2	90	100	70	70
2 4			70	90
2 7			90	100

CONCLUSIONS

1 Phenol and o , m and p cresol produce in rabbits, cats and rats the typical signs of poisoning, described correctly by earlier investigators

2 The degree of toxicity of orally administered phenol appears to be determined primarily, if not wholly, by the absolute amount of phenol present in the stomach, dilute and concentrated preparations showed no marked differences in toxicity

3 The extent of absorption of phenol from the skin appears to be determined

primarily, if not wholly, by the magnitude of the skin area exposed and not by the concentration of the solution in water, within the limits investigated (7, 75 and 95 per cent by volume).

4. Ten-day-old rats are most susceptible to phenol poisoning (oral and cutaneous), 5-week-old rats are least, while adult rats take an intermediate position.

5. The statements of other investigators that *p*-cresol is more toxic than phenol or either of the other cresols have not been confirmed. All these compounds are in the same general range with respect to their toxicity. *m*-Cresol, however, is definitely the least toxic.

6. Soap and plenty of water is a very effective agent for removing phenol from the skin; a 50 per cent solution of alcohol in water is equally satisfactory if it is not applied over a large area of the body surface for a prolonged period (alcohol is absorbed by the skin and may augment the collapse induced by phenol).

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CLINICAL TESTS OF CELLULOSE ACETATE PHTHALATE AS AN ENTERIC COATING[1]

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Although many experiments have been performed in testing the enteric properties of various types of preparations (1–10), it is commonly believed that none of the available preparations at present are completely satisfactory   Consequently, the four chemical characteristics of cellulose acetate phthalate[2] which indicate its possible usefulness as an enteric material have led to the examination of its behavior under clinical tests   The four properties referred to are as follows 1) it is extremely insoluble in acid solutions, 2) it is rapidly soluble in dilute alkaline solutions, 3) it will form a coating film, and 4) it is non-toxic   These properties contain the advantages needed for an enteric coat and the results cited below indicate that the material has considerable promise

In a thorough clinical test of an enteric coating material, two questions arise First, does the coating always disintegrate in the gastro-intestinal tract?   Second, does the coating ever disintegrate in the stomach?   These questions have been attacked separately, and the rationale of this approach is given briefly

DISINTEGRATION TIME   A useful enteric coat must disintegrate in the intestine   In practice it is extremely difficult to determine exactly the disintegration site of a coated preparation   However, by radiographic techniques it is relatively easy to determine whether disintegration has occurred   Thus, to answer the first question, the times required for the disintegration of capsules containing $BaSO_4$ and $BaSO_4$ tablets coated in each case with cellulose acetate phthalate have been measured by means of periodic radiographs   The subjects used in these series of tests were for the most part normal, male, medical students   Due to limitations of time, it was necessary in many cases to discontinue the experiments after about 8 hours   Consequently, the answer to this question is not given as a simple affirmative, but instead is given as the percentages of the capsules or tablets which had disintegrated in the 8 hour period   No proof was obtained in this part of the study that disintegration had occurred in the intestine

DISINTEGRATION SITE   Having by these radiographic examinations determined the approximate time after ingestion at which capsules or tablets disintegrated in the various subjects, a different technique was employed to obtain an answer to the second question   As before, because the subjects were rarely

available for protracted periods of observation, most of the experiments were limited to about 8 hours.

The procedure briefly was to record the position of the capsule or tablet by fluoroscopic examinations repeated at short intervals. When the capsule or tablet had shifted from a position which had been previously identified as within the stomach, a drink of BaSO₄ preparation was administered and a radiograph made. By demonstrating that the capsule or tablet was either (a) intact and outside the stomach, (b) intact and within the stomach at the end of the experiment, or (c) in a few cases lost by disintegration in the period between examinations, a reasonably satisfactory answer to the question was made. Of course, if the capsule or tablet had disappeared between examinations, no conclusions were possible as to the site of disintegration. If the coated preparations were intact and within the stomach at the termination of the test, the ability of the coating to withstand exposure to gastric fluids for extended periods was demonstrated. The observation of the capsules or tablets outside the stomach is proof of the completion of the first requirement of an enteric coat, viz., that it does not break down in the gastric fluids. Outlining the stomach lumen with a drink of a barium meal and recording on a radiograph that the capsule or tablet was not in the stomach gave objective evidence that the preparation had passed through the stomach and was still intact. Unfortunately, after ingestion of the barium meal, the radiopaque material becomes spread along the gastrointestinal tract and prevents the observation of the subsequent disintegration of the coated preparation. However, this lack is not important because the disintegration characteristics of the preparation had already been established in the disintegration time studies.

DISINTEGRATION TIME STUDIES. In an exploratory series, radiographs were taken on a group of 15 subjects, mostly normal, male medical students, at the times after ingestion indicated in table 1. Each subject took two capsules containing BaSO₄ after breakfast or after lunch as indicated. Radiographs were made, with the subjects prone, at hourly intervals after ingestion of the capsules. In the table some effort was made to indicate the location of the capsule—that is, whether it remained in the stomach or had apparently moved into the intestine. In the light of the studies to be reported below it is probable that many of the cases marked as "I" indicating that the capsule had moved into the intestine were erroneous guesses.

Several interesting points arise from consideration of the data in table 1. Certain subjects have a much more rapid disintegration time than others, for example, in each of three tests on J. E. both tablets had disintegrated three hours after ingestion, whereas for J. P. seven hours were required in each of two tests. In general, most of the tablets disintegrated in the fourth and fifth hours. Thus, 6 per cent of the capsules disintegrated in the fourth hour, an additional 48 per cent disintegrated in the fifth hour and by the end of the sixth hour 72 per cent had disintegrated. From these data, a schedule of two examinations at the fourth and at the eighth hours, respectively, was chosen for later studies instead of the hourly schedule.

In table 2 are given the data on the disintegration times observed for various enteric preparations as indicated   This table also includes the data from table 1

TABLE 1
Disintegration time

Data on patients ingesting cellulose acetate phthalate enteric capsules containing BaSO₄ and showing the hours after ingestion when disintegration occurred
Lot #14564 3 minim enteric capsule Gelatin Products Co

PATIENT	UNIT NO	DATE	TOTAL NO OF FILMS	1	2	3	4	5	6	7	WHEN TAKEN*
HGH	49059	3/10/42	2	XX†		XX					AB
		3/26/42	2	II		XP					AB
		4/ 6/42	2	SS			II				AL
		4/ 7/42	1		SS						AL
VT	23781	4/ 7/42	4	SS	SS	SS	SS				AB
		4/ 8/42	4		SS	SS	IX	XX			AB
		4/ 9/42	5		SS	SS	SS	II	PP		AB
		4/18/42	6	SS	SS	SS	II	II	XX		AL
JL	180727	4/10/42	2			SX	XX				AB
		4/11/42	3	SS	II	XX					AL
		4/18/42	3	SS	SS	XX					AL
FB	106422	4/11/42	6	SS	SS	SS	II	IX	XX		AL
		4/18/42	5	SS	SS	SX	XX	XX			AL
JB	180715	4/11/42	5	SS	SS	SS	II		XX		AL
		4/18/42	6	SS	SS	SS	SS	PP	PP		AL
DB	180716	4/11/42	6	SS	SS	SS	II	IX	XP		AL
		4/18/42	6	SS	SS	SS	II	II	XP		AL
RC	180722	4/11/42	6	SS	SS	II	XP	XX			AL
		4/18/42	6	SS	SS	SP	SI	PP	XX		AL
RG	180732	4/11/42	6	SS	SI	SI	II	XX			AL
		4/18/42	6	SS	SS	SI	SI	XP	XX		AL
GH	180736	4/11/42	5	SS	SS	SI	II	XX			AL
JP	180748	4/11/42	7	SS	SS	SS	SS	SS	IX	XX	AL
		4/18/42	7	SS	SS	SS	SS	SS	SX	XX	AL
DS	180756	4/11/42	5	SS	SS	SS	SS?	XX			AL
		4/18/42	5	SS	SS	SS	SS?	XX			AL
EW	180761	4/11/42	6	SS	SS	SS	SS	IX	XX		AL
		4/18/42	6	SS	SS	SS	II	PP	PP		AL
BW	180765	4/11/42	6	SS	SS	II	IP	XP	XX		AL
LS	180754	4/18/42	5	SS	SS	SS	SI	XX			AL
DW	180764	4/18/42	5	SS	SS	SS	IP	XX			AL

* AB = after
† Key S =
tegrated, X =   ’ = capsule partially disin

Using tablets coated with cellulose acetate phthalate as well as enteric capsules, seven series of tests were made on five products   83 patients were followed in a

total of 226 tests. The number of capsules or tablets which disintegrated hour by hour is shown up to the eighth hour. Since many of these tests were run only at the fourth and eighth hours, preparations which disintegrated in the interval appear under the eight-hour column. Nevertheless, the clear-cut tendency for the disintegration to occur most frequently in the fourth to sixth hours, inclusive, is shown. A total of 15 of the 166 capsules or tablets had not disintegrated at the termination of the experiment (eight hours).

TABLE 2

Data on disintegration times of various enteric preparations as shown by radiographic examinations

DATE	MATERIAL TESTED	NO. PATIENTS	NO. TESTS	NO. DISINTEGRATED IN HR.								NO. NOT DISINTEGRATED AFTER HR.							
				1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
3/10–4/10	A	3	16	2	3	3	3	1	4	2									
7/18?	A & B	18	20				4	17	6	6									2
7/25	A	11	14			1	15	1			2					1			2
7/30	C	12	24								2								22
8/5	D	12	16	19 of 24 in 12 hrs.; 21 of 24 in 16 hrs.															
8/6	D	12	24				.	1			10				23				13
8/1	E	13	21				10		2		6								6
4/18	F	12	66			2		8	10									6	
4/11	F	12	62			2	2	10	8								2		
8/11	G	14	27				7				14								5
	H	12	24																24
Totals*	5	83	226	2	3	8	41	37	30	8	22								15
Totals†	2	36	64								12								

A—Squibb, 1½ gr. BaSO$_4$, C.A.P. coated tablets.
B—Squibb, 3 gr. BaSO$_4$, C.A.P. coated tablets.
C—Squibb, 1½ gr. BaSO$_4$, "regular" coated tablets.
D—Squibb, 3 gr. BaSO$_4$, "regular" coated tablets.
E—Strasenburgh, 5 gr. BaSO$_4$, C.A.P. coated tablets.
F—Gelatin Products Co., 3 m. BaSO$_4$ in oil, enteric capsules (14564).
G—Gelatin Prod. Co., 3 m. BaSO$_4$ in oil, enteric capsules (17108).
H—Gelatin Prod. Co., 3 m. BaSO$_4$ in oil, enteric capsules (16456).
Footnote: Dosage not same in all cases so no math. check.
* For C.A.P. enteric preparations (omitting H).
† For "regular" enteric preparations.

Using two sizes of "regular" enteric coated tablets, three tests made on 36 patients involved 64 radiographic examinations. In this series only 12 of 72 tablets had disintegrated at the end of eight hours; however, 21 of 24 had disintegrated in 16 hours.

The Gelatin Products Company submitted three lots of enteric capsules, of which one lot (II, table 2) was completely unsatisfactory. Of the other two lots, one (G) showed 25 per cent disintegration in four hours and 82 per cent in eight hours. The other (F) showed 26 per cent disintegration in four hours and 100 per cent in eight hours. E. R. Squibb and Sons submitted two lots of cellulose

acetate phthalate coated tablets   From one lot 92 per cent had disintegrated in four hours and no more in eight hours   The R J Strasenburgh Company submitted one lot of cellulose acetate phthalate coated tablets of which 42 per cent disintegrated in four hours and 79 per cent in eight hours   In comparison the "regular" enteric coated tablets prepared by Squibb showed 8 and 42 per cent, respectively, disintegration at eight hours in two tests, and in a third test 79 per cent at 12 hours and 88 per cent at 16 hours

The answer to the first question can now be made   Does the coating always disintegrate in the gastro-intestinal tract?   Tests on five products have shown that 79 to 100 per cent of the capsules or tablets disintegrated within eight hours after ingestion   Enteric preparations using cellulose acetate phthalate are judged to be satisfactory in their disintegration time characteristics

SITE OF DISINTEGRATION   In these experiments capsules or tablets prepared with cellulose acetate phthalate were administered before lunch to groups of normal, male medical students   Each subject received one capsule or tablet   About an hour after ingestion the fluoroscopic examinations were begun   The capsule or tablet containing $BaSO_4$ was plainly visible in the fluoroscope, and at each examination the approximate location of the shadow was noted relative to whatever vertebra represented its vertical position in the abdomen   Since two or more examinations usually were made before the capsule or tablet had left the stomach the location of this organ was established   When the capsule or tablet had moved from the site recognized as being within the stomach and was judged to be in the intestine, a small drink of $BaSO_4$ was given to outline the stomach and a radiograph made   In this way positive evidence was obtained that the capsule or tablet had not disintegrated in the stomach   Six such experiments were carried out on preparations furnished by three manufacturers   In figure 1 an illustration is given of the type of record obtained which shows the stomach outlined by the barium meal and the tablet intact and outside the stomach

In table 3 is given the summary of the results of the fluoroscopic examinations A total of 87 subjects were studied and 526 fluoroscopic examinations were made In 45 cases the tablets were demonstrated intact and outside the stomach   All but four of these cases were reported in the third to sixth hours inclusive, and 33 of them were found in the period $3\frac{1}{2}$ to $5\frac{1}{2}$ hours after ingestion   In 29 cases the experiment was discontinued when the capsule or tablet was intact and still inside the stomach   21 of these cases were between the fifth and eighth hours, respectively   In 12 cases the capsule or tablet disappeared between fluoroscopic examinations, in 11 of the 12, this occurred prior to the fifth hour   Despite the frequent fluoroscopic check ups, it is our opinion these preparations tended to disintegrate so promptly on leaving the stomach that the site of their disintegration cannot be established   In one case a capsule was shown to disintegrate within the lumen of the stomach

Of the capsules and tablets shown radiographically to be intact and outside the stomach only four were so demonstrated before the third hour   19 were found before the fifth hour, and 20 by the end of the sixth hour   One was found in the

seventh and one in the tenth hour after ingestion. Apparently the average time that these capsules and tablets were retained in the stomach was about four hours. However, the fact that 16 tablets were still intact and within the stomach in the sixth, seventh, and eighth hours after ingestion, emphasizes the prolonged intervals in which enteric preparations may be retained within the stomach. Retention in the stomach up to eight hours gave no indication of a tendency for the cellulose acetate phthalate enteric preparations to disintegrate as a result of the chemical and mechanical effects of the gastric juice and of the stomach's muscular activity.

FIG. 1. The stomach is seen at the center right of the reproduction of the radiograph. The body of the stomach and the pylorus are outlined by the barium meal. Some of the barium meal has entered the duodenum and can be seen both in the descending and transverse portions. The barium sulphate tablet coated with cellulose acetate phthalate is clearly seen at the left center in the descending third of the duodenum, intact and outside the stomach.

In one case, shown in fig. 2a, a capsule was observed in the act of disintegrating within the stomach. This patient was a female medical student whose stomach was placed low in the abdomen. The capsule had been observed near the level of the second sacral vertebra body during a period of approximately $6\frac{1}{2}$ hours preceding its disintegration. The subject was quite hungry at the time disintegration occurred and marked peristaltic waves were evident. When beginning disintegration was noted the subject was placed prone on the x-ray table and a radiograph made. In this position the subject's stomach appears opposite the third lumbar vertebra. Flecks of $BaSO_4$ may be seen clearly outlining the greater curvature of the stomach. And in fig. 2b, taken about ten minutes later, the disintegration was complete. Following a drink of $BaSO_4$, it was discovered that the duodenum seemed to stretch up from the pylorus and toward the right

TABLE 3

Data on location of cellulose acetate phthalate enteric preparations by hours

DATE	PROD		PATIENTS	EXAMS	1	2S	3	3S	4	4S	5	5S	6	6S	7	7S	8	10	11	12	TOTALS
11/7	A		13	63									1S	2S*	1S	1S	3S	1I	1S	1S	
11/14	B		14	75			1G 1I	1I	2I 3G	2G	1I 1S	1S	1I 1G								
11/21	B		16	84			2I 1G	1I	3I	1G 1I	3I	2I 1S	1I 1S	1I 2S	1I 1S						
11/28	C		16	116	1I		1G	1I 1S	2I	1G 2I	3I	1I	1I	2S							
12/5	C		13	109		1G		1I	1I	1I	1I	1I 1S	1I 1S								
12/12	A		15	70			1S	2I 2S	1S	1I	1I	1I 1S									
			—	—																	
			87	526																	
I—Intact and outside stomach					1		3	6	8	5	9	5	5	1	1			1			45
S—Intact and inside stomach							1	3	2			4	3	7	2	1	3		1	1	29
G—Disappeared between exams						1	3		3	4	1		1								12

* Capsule disintegrated in stomach
A—Squibb, 1½ gr $BaSO_4$, C A P coated tablet (3950-L-1).
B—Gelatin Products Co, 3 m $BaSO_4$ in oil, capsule (17108).
C—Strasenburgh Co, 5 Gr $BaSO_4$ tablet, C A P. coated

upper quadrant. Portions of the barium meal which were ejected into the duodenum were seen to reflux just above the pyloric cap. It is probable that in this patient some regurgitation of alkaline intestinal contents had occurred and that the alkalinity in the stomach became sufficient to dissolve the enteric capsule. It is our opinion that if the other capsules and tablets which disappeared in the intervals between fluoroscopic examinations had disintegrated in the stomach, some indication of this site should have been noted from evidences of BaSO$_4$ particles within the stomach. Since no such evidences were observed we believed that the disintegration in these cases probably occurred in the intestine.

2a   2b
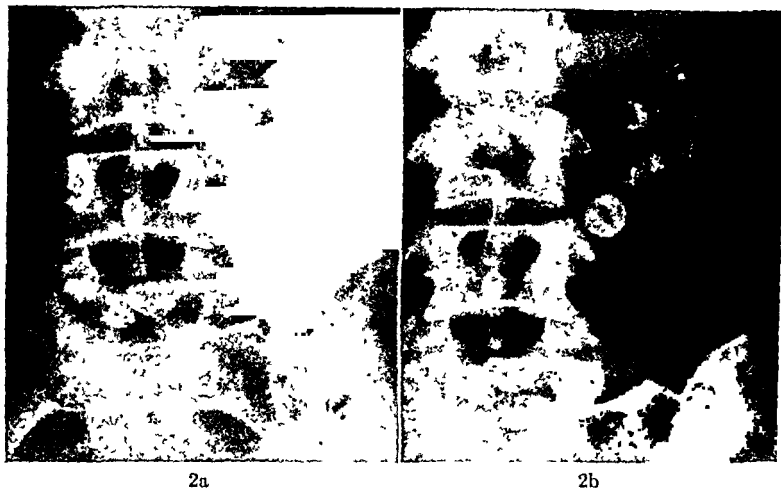
Fig. 2a. The disintegrating capsule is shown to the right of the third lumbar vertebra. Some of the material which has escaped from the capsule outlines the smooth curve of the lower part of the body of the stomach.

Fig 2b. This roentgenograph was taken about ten minutes later than Fig. 2a. The capsule has completely disintegrated now and flecks of the barium sulphate are evident here and there in the lower part of the stomach.

This opinion is strengthened by the results of a limited series of laboratory tests in which each of the preparations used was subjected to artificial gastric juice and to artificial intestinal juice. Most of these cellulose acetate phthalate preparations would withstand the action of artificial gastric juice for 24 hours, although in one case disintegrations in 16 to 20 hours were observed. Placed in intestinal juice they disintegrated in 30 to 90 minutes. If they were treated for periods of a few hours with artificial gastric juice, the disintegration in subsequent exposures to the artificial intestinal juice was very prompt—usually within 30 minutes.

The results of this study indicate that cellulose acetate phthalate coated tablets and enteric capsules possess satisfactory enteric characteristics. In 79 to 100 per cent of trials, disintegration occurred in the gastro-intestinal tract

in the 8 hours following ingestion There is evidence that disintegration may occasionally take place in the stomach, one such disintegration was observed The bulk of the evidence however indicates that in vivo the tablets and capsules coated with cellulose acetate phthalate resist the action of gastric juice for long periods and that, in a majority of the cases observed, such preparations may be found intact and outside the stomach By combining the results of the two phases of this study, the inference is drawn that the cellulose acetate phthalate coating withstands exposure to gastric juice but disintegrates in the intestine

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THE CHRONIC TOXICITY OF CELLULOSE ACETATE PHTHALATE IN RATS AND DOGS[1]

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INTRODUCTION. The literature on the toxicity of phthalic acid and its derivatives is limited to a few reports on acute toxicity studies (1–7). No reports are available on the chronic toxicity of cellulose acetate phthalate. This compound has certain properties which suggest that it may be useful as an enteric coating material. Since enteric coated preparations might be taken by patients over prolonged periods of time, it is necessary to establish the innocuous character of cellulose acetate phthalate in a long term feeding experiment.

Cellulose acetate phthlate has been fed to groups of rats and to dogs for a period of a year without harmful effects. It is interesting to note that the albino rat will tolerate a diet containing 30% of cellulose acetate phthalate whereas incorporating more than 20% of such an inert compound as inulin will produce excessive diarrhea. In general, cellulose acetate phthalate seems to be remarkably inert as a compound of the diet.

TOXICITY IN RATS. In a preliminary experiment, groups of five rats each were placed on various levels of cellulose acetate phthalate in the diets. Rats receiving 5 and 20 per cent of cellulose acetate phthalate showed no obvious interference with health, although the group receiving 20 per cent grew somewhat more slowly than those receiving 5 per cent. An attempt to include 40 per cent of cellulose acetate phthalate in the diet produced so marked a diarrhea and such rapid weight loss that this level was reduced after one week to 30 per cent. The rats receiving 30 per cent cellulose acetate phthalate grew steadily, although not so well as those receiving 20 per cent. Similar control groups were set up in which comparable dietary levels of inulin were used. Rats receiving 5 per cent inulin in their diet grew somewhat better than the control rats. Rats receiving 20 per cent of inulin grew almost as well as the controls; however, rats receiving 40 per cent inulin developed a severe diarrhea and lost on the average of 15 gm. in the first 4 days, whereupon the level was reduced to 30 per cent inulin. In the succeeding 4 days, the diarrhea continued and an additional 15 gm. average body weight loss was observed. The amount of inulin was then cut to 20 per cent, whereupon the rats grew rapidly and at the end of 5 weeks were almost as heavey as the group fed 20 per cent throughout. It was concluded that a dietary level of 30 per cent of cellulose acetate phthalate is tolerated by rats, whereas 20 per cent inulin is apparently near the maximum level.

These rats were autopsied and on autopsy appeared grossly normal. The

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gastro intestinal tract of rats receiving high doses of cellulose acetate phthalate contained an extremely mucilaginous material of brown color   From the autopsy examinations conducted by Dr Sidney Madden of the Department of Pathology, the following summary is made   The rats' organs were smaller in the groups having the higher concentration of cellulose acetate phthalate in the diet   The cause of this growth retardation may be a reduction of food intake or an interference with the digestion and absorption of food or both   These explanations appeared more likely than one which assumed a positive tissue toxicity for the test compound, since the control animals receiving inulin showed similar growth retardation   The focal pneumonitis seen in several rats is rather common in these laboratory animals   It should be noted that no congestion of the intestine is found in these rats nor is there any evidence of renal injury

It was shown by the preliminary experiment, a) that increasing amounts of cellulose acetate phthalate in the diet increasingly reduced the average growth rate, and b) that in equal amounts cellulose acetate phthalate appeared to be more effective in reducing the growth rate than was inulin   This may be attributed to the peculiar physical character of the contents of the intestine

In the chronic experiment begun in September, 1941, four groups of 20 female rats each were placed at weaning on various dietary levels of cellulose acetate phthalate   From the information gained in the preliminary experiment, these levels were set at 0, 5, 20 and 30 per cent, respectively   The diets were basically a Purina fox chow meal into which the cellulose acetate phthalate was mixed   Food and water were given *ad lib*   The rats were kept in groups of 5 to 7 in large cages   Weights on each animal were recorded weekly   Except for a slight epidemic of pneumonia which appeared in the colony near the termination of the experiment, all of the rats grew well, ate well, and were in excellent condition throughout the experiment   As may be seen from fig 1, the control rats grew well and reached an average body weight of about 220 gm   The rats receiving 5 per cent cellulose acetate phthalate grew nearly as well, although their average weight curve is displaced about 5 gm below the growth curve for the control group   The growth curve for the rats receiving 20 per cent cellulose acetate phthalate is displaced approximately 15 gm below that of the control rats   The group receiving 30 per cent cellulose acetate phthalate grew steadily   However, their body weight averaged some 40 gm less than the corresponding group   The individual rats in each group had considerable variation in growth curves as is customarily observed

The animals were sacrificed in September, 1942, after one year on the diets   They appeared, except for a few animals with pneumonitis, to be normal   At the time of autopsy, weights were obtained on brain, liver, kidney, heart, spleen, stomach and lungs   Sections of each of these tissues were taken from each rat for histological examination   In addition, specimens were removed from the small and from the large intestines

In table 1 are given the average organ weights   For most tissues the weights of the various organs followed closely the differences in average body weights of the 4 groups   Thus, the liver in the control group and the group receiving 5

per cent cellulose acetate phthalate averaged about 7.3 gm.; in the 20 per cent cellulose acetate phthalate group, about 7.0 gm.; and in the 30 per cent cellulose acetate phthalate group, about 5.6 gm.   Only in the case of the stomach weights was any abnormality observed.   Here the control, 5 per cent and 20 per cent cellulose acetate phthalate groups had average stomach weights of 1.2 to 1.3 gm., whereas the 30 per cent group averaged 1.6 gm.   The lung weight for the control group is high, due to the masses of inflamed tissue in a few of these rats.
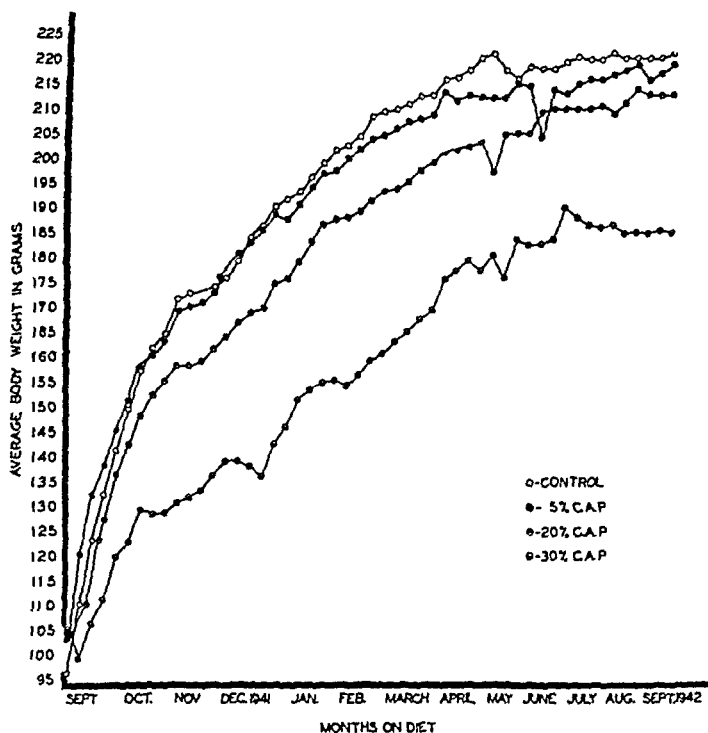
FIG. 1. GROWTH CURVES FOR RATS ON VARIOUS DIETARY LEVELS OF CELLULOSE ACETATE PHTHALATE

The addition of 5 and 20 per cent, respectively, of cellulose acetate phthalate reduced the growth rate slightly; 30 per cent of cellulose acetate phthalate in the diet produced a marked diminution in growth.

When the organ weights are computed on the basis of body weights, i.e., mgm. of organ per 100 gm. body weight, a complete uniformity in the average weight of the organs is found except in the case of the stomach (see table 1, lower part). The average stomach weight for the control and 5 per cent cellulose acetate phthalate group is 5.3 to 5.6 gm.; for the 20 per cent cellulose acetate phthalate group, the average weight is 6.2 gm.; and for the 30 per cent cellulose acetate phthalate group, the average weight is 8.6 gm.   This may be taken as an evidence

of a functional hypertrophy. The rats on the highest cellulose acetate phthalate intakes were receiving less nutritious matter from their diets, as is shown by the lower growth curves. In an effort to ingest sufficient food to satisfy their growth impulse, it may be assumed that these rats habitually over ate and eventually developed a larger and heavier stomach.

The histological examinations were carried out by John R. Carter of the Department of Pathology, his report may be briefly summarized. In general, only non specific pathological findings were drawn from the study of the tissues of the rats receiving various doses of cellulose acetate phthalate. The most striking and consistent lesions were found in the kidney in all groups of animals. These lesions consisted of a diffuse degeneration of tubular epithelium. However, the degree of damage was in inverse relation to the dosage of the drug. Furthermore, the control animals showed similar changes but to a lesser degree. The kidney

TABLE 1

The average weights of various organs at autopsy of female rats on various dietary levels of cellulose acetate phthalate

GROUP	DIET	AVERAGE BODY WEIGHT	BRAIN	LIVER	KIDNEY	HEART	SPLEEN	STOMACH	LUNGS
				Average organ weights					
		gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
A	5% CAP	221	1 48	7 25	1 66	0 75	0 62	1 18	1 38
B	20% CAP	215	1 40	7 02	1 59	0 67	0 55	1 33	1 34
C	30% CAP	187	1 35	5 59	1 55	0 63	0 56	1 61	1 17
D	control	226	1 41	7 26	1 69	0 75	0 66	1 26	1 72
			Organ weights on the basis of body weights (mgm per 100 gm body weight)						
A	5% CAP	221	6 7	33 0	7 5	3 4	2 8	5 3	6 2
B	20% CAP	215	6 5	33 0	7 4	3 1	2 5	6 2	6 2
C	30% CAP	187	7 2	29 0	8 3	3 3	3 0	8 6	6 2
D	control	226	6 2	32 0	7 5	3 3	2 9	5 6	7 6

lesions were found in that part of the kidney where any toxic property of the drug would have little significance. The origin of the abnormalities is not known. However since these abnormalities are not correlated with the dietary levels of cellulose acetate phthalate, it is probable that they did not originate as the result of its ingestion. In general, these examinations give evidence of a very low order toxicity of cellulose acetate phthalate in rats.

TOXICITY IN DOGS. In a preliminary experiment, three dogs were placed on intakes of 1, 4, and 16 gm of cellulose acetate phthalate daily for a period of one month. The cellulose acetate phthalate was incorporated in hamburger and swallowed at a gulp. The dogs gained weight, ate well, no diarrhea developed and they had every appearance of being in good health at the end of the experimental period. Red blood cell counts, hematocrits and icteric indices were determined at the beginning and after 2 and 4 weeks, respectively. Values were

normal in each case. Periodic urine analyses were negative for albumin and sugar. From complete autopsy examinations made by Dr. Sidney Madden, the following summary is taken: a congestion of the mucosal blood vessels of the small intestine is moderate in the dog receiving 16 gm. cellulose acetate phthalate per day, and mild in the dog receiving 4 gm. This blood vessel engorgement may mean irritation, but the irritation is so slight that no hemorrhage or leucocyte infiltration, or other evidence of inflammation has occurred. The urinary tract abnormalities noted in the autopsy protocols of the three dogs can all be attributed to infection, chiefly incidental to catheterization.

In the chronic experiment, six dogs were placed for one year on diets containing various levels of cellulose acetate phthalate as in the preliminary experiment. All of the dogs remained in excellent condition during the entire period. Their appetites were good, and normal feces were uniformly observed. All of the dogs gained some weight, and for reasons unknown the two dogs on the highest dose of cellulose acetate phthalate (16 gm. daily each) gained markedly—about 10 lbs. each. The dogs were sacrificed in October and November, 1942, after about one year on the cellulose acetate phthalate diet. Complete autopsies were performed by Louis J. Zeldis of the Department of Pathology.

A summary of the detailed autopsy reports showed certain findings occurred with regularity. In each dog, the histological examination of the splenic tissue revealed hemosiderosis and diffuse fibrosis. There were small deposits of hemosiderin in the liver and bone marrow, and sometimes in the lung. Occasional small amounts of slightly basophillic cytoplasm were found in the sinusoids of the liver; these appeared to represent small islands of extramedullary hematopoiesis. Lungs in each case showed a terminal pulmonary congestion from the use of ether as a lethal agent.

On the day of autopsy, the dogs in each case appeared well-nourished, alert and active. The autopsy findings which might indicate evidences of toxicity of cellulose acetate phthalate in these animals were limited to the spleens, where there were considerable deposits of hemosiderin pigment and an associated diffuse fibrosis of varying degree. These findings cannot be entirely interpreted by post-mortem studies; however, they suggested a process of blood destruction. There appeared to be no parallel between the dose of cellulose acetate phthalate and the extent of these changes: the two dogs which received intermediate doses (4 gm. daily) exhibited the most marked alterations.

The splenic changes noted are probably related to factors other than the administered drug. Entirely similar findings have been noted in the spleens of 4 of 6 dogs examined for purposes of comparison. These animals were clinically well and had been subjected to no experimental procedures, although each of them had spent considerable time in the same animal colony as the dogs which had ingested cellulose acetate phthalate. Consequently it is concluded that no toxic effect of the cellulose acetate phthalate administered was revealed in the post-mortem studies.

SUMMARY

1 Four groups of 20 female rats each were fed 0, 5, 20 and 30 per cent cellulose
acetate phthalate, respectively, for a period of one year   The rats on high in-
takes of cellulose acetate phthalate showed a reduction in growth rate which
increased with the dosage   On autopsy, the rats were in good condition and no
abnormalities were observed save that the average stomach weight tended to
increase with higher doses of cellulose acetate phthalate   From histological ex
aminations, no consistent pathological changes were demonstrated   High doses
of cellulose acetate phthalate in the diet tended to produce a mucilaginous char
acter of the material in the intestinal lumen   From these observations it is
concluded that high levels of cellulose acetate phthalate in the diet of rats inter-
fere quantitatively and mechanically with the absorption of food   No toxic
action of cellulose acetate phthalate has been found in rats

2 Three groups of 2 dogs each were fed 1, 4 and 16 gm , respectively, of cellu-
lose acetate phthalate during a period of one year   The dogs remained in ex-
cellent health and condition throughout the experiment and no consistent patho-
logical changes were discovered at autopsy   There is no evidence of any toxic
effects of cellulose acetate phthalate under these conditions

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EFFECTS OF POSTERIOR PITUITARY EXTRACT, OXYTOCIN (PITOCIN) AND ERGONOVINE HYDRACRYLATE (ERGOTRATE) ON UTERINE, ARTERIAL, VENOUS AND MATERNAL EFFECTIVE PLACENTAL ARTERIAL PRESSURES IN PREGNANT HUMANS[1]

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It is well known (1) that the use of oxytocics prior to delivery of the newborn infant is fraught with dangers of 1) intra-uterine asphyxia to the child and 2) traumatic injury to the mother and child. As a result, ergot preparations are rarely administered before delivery. Yet, pituitary preparations are still widely used by some physicians to hasten delivery. Is this good obstetrics? Are the dangers serious and real? Answers to these questions were sought in the present investigation.

METHODS AND TECHNIQUES. In eighteen patients, pregnant 8 to 9 months, the pressure which tends to rupture the uterus and the maternal arterial pressure which irrigates the placenta were evaluated before and after the administration of oxytocics. The patient was usually one whose pregnancy had best be terminated. It is the custom in this hospital to induce labor by the introduction of a catheter into the uterus outside the chorion on the side opposite the placenta. In these patients a soft rubber balloon (condom) was tied over the end of the catheter and a lead bougie was inserted temporarily into its lumen. With the catheter thus stiffened, it was easily introduced into the fundus of the uterus and its position checked by x-ray. The balloon was partially filled with 30 cc. of 0.6 per cent sterile sulfanilamide. The doses and drugs used are those which are commonly given to such patients by many physicians, although some objection to their use is present in some quarters. Drug administrations were separated by periods of 40 minutes or more to permit recovery from previous therapy. Uterine, arterial and venous pressures were recorded optically by means of simple and differential hypodermic manometers previously described (2, 3) (see fig. 1). The differential manometer is constructed to measure the difference between pressures rather than pressure *per se*.

Uterine pressure results from activity of the abdominal and uterine muscles. The force developed by the abdominal muscles acts upon the outside of the uterus and places no rupturing stress upon the uterus. As shown in fig. 1, the force which tends to rupture the uterus was measured with a differential manometer which subtracted the pressure on the outside of the uterus from the pressure on the inside of the uterus. Technical difficulties made it necessary to use the gastric pressure and to assume that this equals the abdominal pressure. Straining of the abdominal muscles should have the same effect upon the gastric and abdominal pressures.

The maternal arterial pressure which irrigates the placenta is the arterial pressure minus the pressure within the uterus which is opposing the inflow of maternal blood. This maternal effective placental pressure has been measured by a second differential manometer where the uterine pressure is led to the front chamber and the arterial pressure is led to the back of the manometer.

RESULTS AND DISCUSSION   Oxytocics increased the intensity, frequency and *speed of uterine contractions*   The intensity of the contractions determines the stress upon the uterus whereas the amount and duration of the fusion of the intense contractions determine the danger of intrauterine asphyxia to the baby Small subcutaneous doses of pituitary preparations (1 minim—0 66 unit) or small sublingual doses of "ergotrate" (1/1280 grain—0 05 mg) increased uterine activity without producing any fusion of contractions   After larger doses, a

TO NEEDLE IN MATERNAL ARTERY

TO BALLOON IN STOMACH

TO BALLOON IN UTERUS
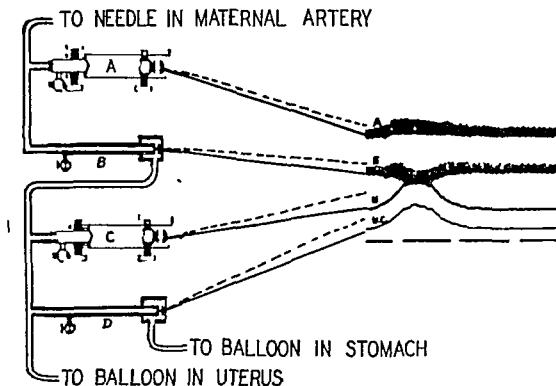
FIG 1 A SOFT RUBBER BALLOON (CONDOM) TIED OVER THE END OF THE CATHETER IS INSERTED INTO THE FUNDUS OF THE UTERUS

   A similar balloon tied over the end of a duodenal tube is introduced into the stomach by way of the nose   The third curve from top U obtained with the simple manometer

moderate amount of interference with relaxation was present in 8 of 10 patients This persisted for 20 to 40 minutes after injections of 2 oxytocic units of pituitary preparations (fig 2) and for 1 hour after administrations of 0 1 mgm of "ergo-trate " When the uterus was hyper reactive, oxytocics as commonly given by many physicians (2 units of ' pituitrin' in one patient and two administrations of 0 1 mgm of "ergotrate" in another patient) caused severe abnormal uterine contractions (tetany) characterized by prolonged and marked interference with relaxation (fig 3) Since such *prolonged* contractions are shown to be hazardous

to the baby (see below), efforts were made to limit the duration of uterine tetany. This was accomplished in five patients by using intravenous injections of 0.66 unit of a pituitary preparation instead of larger subcutaneous administrations which are commonly given by many obstetricians. The severe abnormal uterine contractions from the intravenous injections lasted only 5 to 10 minutes (fig. 4). Using "ergotrate" various degrees of abnormal uterine activity also were obtained in five other patients by four hourly repeated administrations of 0.1 mgm. of the drug.

Stress upon the uterus. In the patients who received 0.66 unit of pituitary preparation intravenously, the uterine pressures occasionally reached 200 mm. Hg during the "bearing down" efforts. However, the maximal pressure developed by the uterus (pressures tending to rupture the uterus) were 75, 80, 85, 88 and 100 mm. Hg. This amounts to a maximal stress of 104 to 140 grams per square cm. of uterine area. Assuming that the contracting uteri were spherical
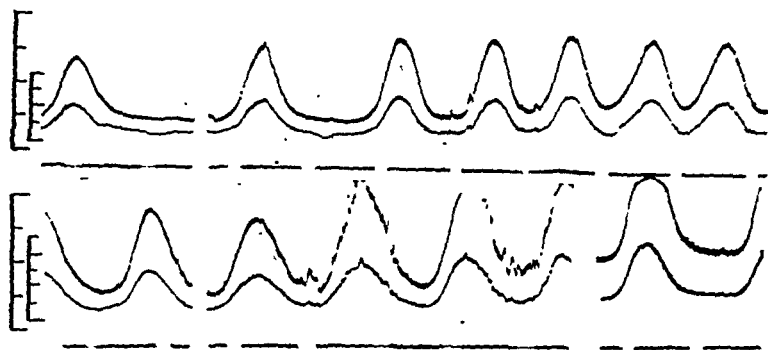
Fig. 2 From above downward: Uterine pressure, uterine contribution to this pressure and time in 1 minute intervals. At break in the upper tracings 0.66 unit of "pitocin" was administered subcutaneously. At first break in the lower tracings 2 units of "pitocin" were administered subcutaneously. At second break, 30 minutes of the record were deleted.

in shape and normal in volume the maximal force tending to rupture the organ was calculated to be from 260 to 350 grams per cm. on the uterine circumference. Since the average thickness of the wall of a full term uterus measures about 5 mm. the maximal tension would be approximately 700 grams per sq. cm. (10 lbs. per sq. in.) of cross sectional area of uterine wall. This exceeds by 100 per cent the maximal values recorded before the injection and by 20 per cent those normally present during the delivery of the head (3). Greater tension, of course, is actually present at sites where the uterus shows a greater radius of curvature and where the wall is excessively thin.

In the patients who received "ergotrate" the maximal pressures developed by the uterus were between 50 and 70 mm. Hg. This corresponds to a maximal tension of approximately 500 grams per sq. cm. of cross sectional area of the uterine wall. The difference between this stress and that produced by pituitary extracts can be explained by differences in dosages.

In gravid patients pituitary and ergot preparations can increase the force of uterine contractions to an intensity equivalent to or slightly exceeding the maximum observed during the delivery of the head   The danger of uterine rupture may be greater, however, since these prolonged maximal contractions and the stress *are forced upon the organ irrespective of its condition*
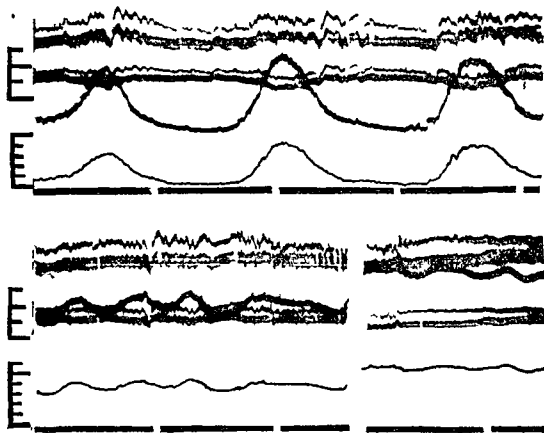
Fig 3   From above downward   Maternal arterial pressure maternal effective placental arterial pressure uterine pressure uterine contribution to the latter and time in 1 minute intervals   Pressure scales are shown in units of 25 mm Hg for the effective placental arterial pressure and in units of 10 mm Hg for the uterine contribution to the uterine pressure

Upper tracings   Seven minutes after intravenous injection of 0 1 mgm 'ergotrate   This increased the force frequency and speed of uterine contractions without significantly changing the uterine tone between contractions

Lower left tracings   Ninety minutes after 1st injection and forty five minutes after 2nd injection of 0 1 mgm   ergotrate

Lower right tracings   Ten minutes later when dangerous abnormal uterine activity was at its height

Intra uterine asphyxia of the baby   Pituitary preparations and ergotrate" even in small doses can produce the type of uterine activity which maintains a high pressure   This causes a pronounced and dangerously prolonged reduction in the maternal arterial pressure which irrigates the placenta (figs 3, 4)   Consequently, maternal blood flow to the placenta is decreased during a prolonged period of time because relaxation between the contractions is incomplete   The action is more prolonged after the administration of pituitary preparations when the subcutaneous route is used   Normal uterine contractions also reduce this

effective placental pressure (3) (fig. 3, upper tracings). Yet, intra-uterine asphyxia is not severe with normal contractions since they are short in duration and the effective placental pressure returns to normal during each relaxation phase.

The risk of fatal intra-uterine asphyxia to the baby is greater when maternal hypotension is present. In one such patient, who received 0.66 unit of "pitocin" intravenously, the fetal heart became faint and irregular and then could not be heard for 3 minutes. At one time the effective placental pressure of this patient was reduced to negative 10 systolic and negative 60 diastolic pressure (fig. 4). This meant that maternal arterial blood not only could not enter the placenta, but that the uterus was actually squeezing blood from the placenta into the aorta as well as into the veins. Fortunately the uterine tetany subsided somewhat and the maternal arterial pressure again supplied blood to the placenta. The extremely low effective placental pressure in this patient can be explained partially by the fact that the mother was hypotensive and partially by the fact
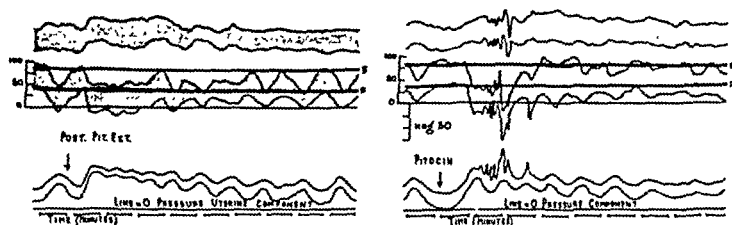
FIG. 4. PLOT OF MEASUREMENTS SECURED FROM RECORDS OF PATIENT
From above downward: Maternal arterial pressure, maternal effective placental arterial pressure with line "S" and "D" indicating average systolic and diastolic values before injection of drug, uterine pressure, uterine component, uterine component base line, and time line which shows 1 minute intervals.

that "pitocin" produces cardiac weakness and lowers the arterial pressure in man (4). These cardiovascular effects prevented the arterial pressure rise which should have resulted from the greater resistance in the uterine vessels and from the increased venous return from the squeezed placenta. This rise, which accompanies normal contractions (3) usually occurred after administering the whole extract. Evidently the action of the vapo-pressor principle frequently masked some of the cardiovascular effects of "pitocin." The rise also occurred after administering 0.1 mgm. of "ergotrate" because the dose of the drug does not directly influence the blood pressure.

Severe intra-uterine asphyxia of the baby was suspected in one patient where where was no serious reduction in the effective placental pressure (see fig. 3). The fetal heart became faint, very slow and irregular as the abnormal uterine contractions appeared. Measures to relieve these signs of intra-uterine asphyxia promptly restored fetal heart tones to normal. The absence of any serious reduction of the effective placental pressure in this patient, when the intra-uterine pressure has been markedly elevated, is due to the fact that the maternal arterial pressure was increased by 60 mm. Hg. This rise in maternal blood

pressure contributed in maintaining effective uterial pressure to the placenta and was without doubt the result of excessive pain, anxiety and distress associated with severe uterine activity   Constriction of the maternal blood vessels must have been present   If intra uterine asphyxia of the baby was present the explanation could be that the vessels supplying the uterus were sufficiently constricted so that the normal effective placental pressure failed to provide adequate blood flow to the placenta   Another possible explanation could be that the high *sustained* intra uterine pressure might empty the placental sinusoids (intervillous spaces)   Any such marked reduction of the quantity of maternal blood in the placenta could limit seriously internal respiration between maternal and fetal blood due to the reduction of the tissue interface

Investigations of the maternal venous pressures supply data concerning flow of blood from the maternal placental sinusoids as shown in fig 5   The maternal
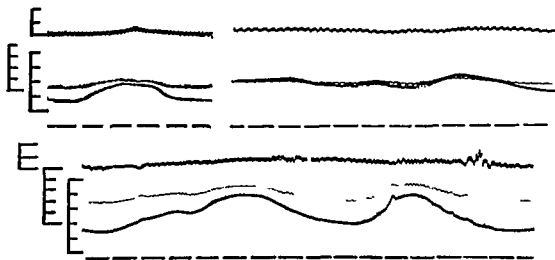
Fig 5 From above

venous pressure increases until uterine pressures of 18 to 23 mm Hg are present   Further elevations in the uterine pressure are without further significant effects upon the venous pressure   These data show that venous outflow from the uterus is increased until the uterine pressure approaches 20 mm Hg and that greater uterine pressures fail to produce further significant increases in venous outflow   Therefore, uterine pressures of about 20 mm Hg either nearly empty the maternal placental sinusoids or create increased resistance to venous outflow   Each of these possibilities shall be discussed

It appears doubtful that the maternal placental sinusoids are nearly emptied since intra uterine asphyxia of the baby was not observed in patients with prolonged uterine pressure of only 20 mm Hg   In addition to this investigations by Barcroft and Rothchild (5, 6) upon rabbits and of Hansen (6) upon humans have shown that near the end of gestation as much as one sixth of the maternal

blood is in the uterus. To empty the placental sinusoids would involve an auto-transfusion of over 1 liter of blood from the uterus during each contraction. The amount appears unreasonably large in view of the fact that the observed maximal rise of maternal venous pressure was 10 mm. Hg.

It is more probable that uterine contractions create resistance to venous outflow from the placenta. This could occur by partial collapse and compression of the uterine sinusoids. As the uterine pressure rises, it adds itself to the blood pressure of vessels within the uterus. This increases the pressure gradient and the flow from the uterine sinusoids to the veins. In turn collapse of these sinusoids would be prevented by an increased blood flow from the intervillous spaces. Yet this increased flow can occur only as a result of some decrease in the net pressure distending the sinusoids. Reduction in this pressure would decrease the size of the sinusoids and create resistance to the flow of blood through them. Such a mechanism would allow venous outflow from the uterus to increase until the pressures were approximately the same in the uterus and in the sinusoids. It also would restrict outflow since further elevations of uterine pressure would compress the sinusoids and hinder venous outflow. This would provide protection of the fetus against serious reduction of tissue interface between maternal and fetal blood. Probably of equal importance, it would protect the mother from receiving an enormous autotransfusion during each uterine contraction.

The observation that the maternal venous pressure rises 10 mm. Hg during uterine contraction (fig. 5) substantiates earlier observations (7, 3, 6) that uterine contractions force blood from the large uterine vessels and sinusoids into the general circulation. This rise of venous pressure explains the absence of any maternal cardiac slowing during uterine contractions. Otherwise, reflex bradycardia would occur and limit the elevation of the arterial pressure occasioned by the increased peripheral resistance resulting from the contraction of the uterus on its blood vessels. This rise of arterial pressure (3) (fig. 3, upper tracings) serves the useful purpose of minimizing the reduction of the effective placental pressure during uterine contractions.

CONCLUSIONS

1. Pituitary and ergot preparations can increase the force of uterine contractions to an intensity equivalent to or slightly greater than that normally present during delivery of the head. However, the danger of uterine rupture may be greater since these prolonged maximal contractions and the stress *are forced upon the organ irrespective of its condition.*

2. The stress placed upon the wall of the uterus is approximately 700 grams per square centimeter of cross-sectional area.

3. Commonly used doses of pituitary and ergot preparations cause abnormal uterine activity in some patients. This abnormal activity, characterized by prolonged interference with relaxation, seriously reduces the maternal effective placental arterial pressure and causes intra-uterine asphyxia of the baby. This danger is greater when the patient has hypotension or when oxytocin is used in place of the whole posterior pituitary extract.

    4 Some mechanisms which prevent ischemia of the placenta are described and discussed.

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DINITROPHENOL CATARACT: PRODUCTION IN AN EXPERIMENTAL ANIMAL[1]

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In 1933 Cutting, Mehrtens and Tainter (1) reported the use of dinitrophenol (2:4 dinitrophenol) in man as a weight reducing agent. A later report by Tainter, Stockton and Cutting (2) covering a series of 113 patients under dinitrophenol therapy showed very satisfactory weight loss in approximately 90% of the cases. These studies on man were made after thorough investigations on the effect of this agent upon animals in relation to changes in metabolic rate (3) and toxicity studies during long repeated administration (4).

Both Tainter and associates (2) and officials of the American Medical Association (5) warned against possible damage that might result from indiscriminate and repeated use of this agent. These warnings were proven correct when during the following 2–3 years numerous reports of various types of toxic and fatal reactions due to the use of dinitrophenol were published.

One of the most important of the undesirable reactions observed in patients using dinitrophenol for weight reduction was the development of cataracts as first described by Horner et al. (6) in 1935. Numerous reports of cataract formation were published in the following year, and in an excellent review of the subject Horner (7) estimates the incidence of cataract formation at 0.86 per cent of those taking dinitrophenol for obesity.

Soon after the observation was made that cataract might develop during or following the use of dinitrophenol in man, attempts were made by several investigators (8) to produce cataracts in experimental animals by administering the drug in the feed as well as by other routes. In none of these studies on rats, rabbits, guinea pigs and dogs was it possible to produce any changes in the lens by the drug. Borley and Tainter (9) added dinitrophenol to various lactose diets which regularly produce cataracts in white rats, but this addition did not alter the time of onset or type of change in the lens which were characteristically noted in rats ingesting the lactose diet alone.

Horner in 1942 states in his summary that "All attempts to produce experimental cataracts in laboratory animals by various and repeated doses of dinitrophenol have been unsuccessful" (7).

During the course of an investigation of a series of drugs upon their effect on the growth of fowl pox in the chick it was observed that within 24 hours after the chick had been placed on a diet containing dinitrophenol opacities of the lens developed. We have followed up this observation with a series of experi-

ments upon the development of cataracts in the fowl ingesting food containing dinitrophenol and we wish to report the results of these studies at this time

EXPERIMENTAL METHODS   *Chicks*   The chicks, White Leghorn or White Rock, were obtained from a commercial hatchery when 1 day old and were kept on Purina Startena feed until ready for use in 2 weeks to 8 weeks

Ducks   The ducks, White Pekin, were bought when 10 days old and were fed a wet mash of Purina Startena for 4 days before use

Administration of drug   The sodium salt of 2 4 dinitrophenol was mixed with the feed in concentrations of 0 05 to 0 25%   This mixture was kept before the chick or duck all the time but the lighting was so arranged that periods of 3 hours duration of light and dark alternated during the experimental period   The duration of any one experiment varied from 3–31 days

Observations   The chicks or ducks were weighed daily after being placed on the drug diet and the eyes examined for the presence of opacities   Daily temperature readings (cloacal) were made on several groups of chicks   There was no increase in temperature during the first four days on the diet

Histological examination   At regular intervals from 5 hours to 30 days individual chicks were sacrificed and the lenses were removed and prepared for microscopical examination, the lenses were fixed in a modified Bouin's fixing solution, imbedded in paraffin, and stained with hematoxylin

RESULTS   In the chicks and ducks on a diet containing 0 25% 2 4 dinitrophenol Sodium, a fine grey opacity usually could be observed in 4–6 hours and by 24 hours this had progressed to a very marked opacity extending over most of the anterior portion of the lens visible through the pupil   In a large proportion of the lenses it was possible to observe a dense opacity in the posterior part after 24 hours on the drug diet   Upon continued feeding of the drug the changes in the anterior portion of the lens began to subside with a persistence or increase in the opacity in the posterior portion

In the chicks on a lower concentration of the drug in the diet the time of development of the opacities was delayed and in many instances no opacities were observed in chicks on a diet containing 0 05–0 15% dinitrophenol

In Table I data are presented relative to the concentration of drugs in the diet, number of chicks or ducks showing gross opacities during the feeding period and remarks relative to weight changes during the drug feeding period

MICROSCOPIC EXAMINATION OF LENS SECTIONS   Upon microscopic examination of lens sections taken after 4–5 hours on the drug diet (25%) a fine granular and vacuolar change was observed in the anterior portion of the lens fibers in the chick   After 24 hours feeding many small and large vacuoles, each extending across one or more lens fibers, could be seen   There was usually a characteristic pattern to the distribution of the lesions, with the vacuoles occurring in 2 or 3 definite levels in the anterior portion of the lens   There was a band of large vacuoles in the fibers just beneath the anterior capsular epithelium, a little deeper there was a band of smaller vacuoles in most of the fibers, and then at a level about one-third of the distance to the center of the lens there was a band of very fine vacuoles (see figs 1, 2, 3, Plate I)

After 24–48 hours on the drug there was great tissue destruction in the posterior portion of the lens   The change in the posterior portion was very different

from that in the anterior portion where in most cases the normal structural pattern of the fibers remained (see figs. 4, 5, Plate I).

Sections of lenses removed from chicks after 4–28 days on the drug diet showed marked changes. The lesions which were present in the anterior portion of the lenses began to resolve so that by 6–8 days on the drug the vacuoles had disappeared but an abnormal pattern of the fibers persisted. The lesions in the posterior part of the lenses gradually changed from a diffuse destructive type

TABLE 1

The effect of 2:4 dinitrophenol in the feed upon the lens and weight of chicks and ducks

D.N.P. CONC.	ORIGINAL WEIGHT	NUMBER OF ANIMALS SHOWING GROSS OPACITIES OVER NUMBER OF ANIMALS ON DRUG DIET ON ANY GIVEN DAY DURING EXPERIMENTAL PERIOD									FINAL WEIGHT CHANGE
		1	2	3	4	5	6	7	8–14	31	
Chicks											
%	grams										%
.25	256	11/12	11/12	10/10							−10
	380	5/6	5/6	5/5	4/5						−17
	527	3/3	3/3		3/3			3/3	3/3		−20
	291	9/12	8/12	9/11					9/9		0
	160	10/12	9/11		9/11		8/10				0
	228		5/5			4/5			4/4		+20
	400		7/7		7/7	6/6	4/5	3/5	3/3		0
*	410		7/7		6/6	6/6	5/5		5/5		−15
.15	60	15/20	17/20	12/16	8/13			1/9	0/7		+20
	369	6/10	5/10	3/9	6/9		3/8		0/8	0/6	+66
	296	3/12	4/12	2/12			3/12		10/12		0
.1	264	7/10	7/10		2/10			5/8			+32
	149	0/10	0/10	1/10							+10
.05	160	0/10	0/10	0/10							+15
Ducks											
.25	110	7/7	6/6	6/6	5/5			4/4	3/3		+10

* This group of chicks received riboflavin (0.35 mgm./100 gram weight intra-muscularly) every 48 hours in addition to D.N.P.

to a cyst-like lesion after 8–10 days on the drug and this persisted as long as the duration of our experiments (see figs. 1–5, Plate II and figs. 1, 2, Plate III).

Ducks. The histological changes which were observed in the lenses of the duck on the drug diet were so similar to those observed in the chick that no attempt need be made to differentiate them. Photomicrographs of lenses taken after 24 hours on the diet are shown in figures 3 and 4, Plate III.

EFFECT OF RIBOFLAVIN ON DINITROPHENOL CATARACTS. Day et al. (10) have reported cataract production in rats on riboflavin deficient diets and they were able to prevent the extension of the cataractous changes by injection of riboflavin into rats while on the deficient diet.

We gave chicks riboflavin (0 35 mg /100 grams) intramuscularly every 48 hours, beginning 24 hours before the chicks were placed on the dinitrophenol diet without altering in any way the usual development of opacities or the typical histological changes   Lens sections taken from chicks receiving dinitrophenol alone and dinitrophenol plus riboflavin are shown in figures 2 and 3, Plate II

EFFECT OF OTHER NITROPHENOLS   *2 6 dinitrophenol*   This compound when given in a 0 25% mixture failed to produce any change in the lens although the chicks lost 25% in weight during the 6 day feeding period, with a 0 5% concentration in the diet two of thirteen chicks developed a very fine grey opacity in the anterior part of the lens which was present only on the second and third day of feeding

2 4 dinitro,6-aminophenol   This compound, picramic acid, when added to the diet in concentration of 1% produced lesions very similar to those in chicks receiving a diet containing 0 25% dinitrophenol

DISCUSSION   Although the relatively high incidence of cataract formation (86%) in people taking dinitrophenol for weight reduction indicates that there is a definite relation between the ingestion of the drug and the subsequent change in the lens, there have been no previous experimental studies reported which show such a cause effect relationship

These experiments show that, at least in the young fowl, 2 4 dinitrophenol, when fed in sufficient amounts, routinely produces changes in the lens which are remarkably similar to those occasionally observed in man   The description of the changes in the lens of man given by Horner (7) is quite similar to the changes observed in chicks in that there is first a fine grey opacity in the anterior subcapsular portion of the lens, no involvement of the lens nucleus, and a more marked destructive lesion in the posterior portion of the lens

There is at present no explanation regarding a difference in effect of 2 4 dinitrophenol in different subjects which would account for the production of cataracts in man and fowl but not in rats, rabbits, guinea pigs and dogs

It is of interest to note that with the fowl the 2 4 dinitro substituted compound is more active in the 2 6 substituted phenol

SUMMARY

The production of cataracts in an experimental investigation with dinitrophenol (on fowl) has been successful

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EXPLANATION OF PLATES

Photomicrographs of lens sections taken from chicks or ducks on diet containing 2:4 dinitrophenol. Magnification. ×120; original plates reduced from 6 x 9 to 4 x 6 inches.

PLATE I

FIG. 1. Anterior portion of a lens from a chick receiving a diet containing 0.25 per cent 2:4 dinitrophenol for one day.

FIG. 2. Anterior portion of a lens from a chick receiving a diet containing 0.25 per cent 2:4 dinitrophenol for two days.

FIG. 3. Anterior portion of a lens from a chick receiving a diet containing 0.1 per cent 2:4 dinitrophenol for two days and 0.25 per cent 2:4 dinitrophenol for two days.

FIG. 4. Posterior portion of a lens from a chick receiving a diet containing 0.15 per cent 2:4 dinitrophenol for two days.

FIG. 5. Posterior portion of a lens from a chick receiving a diet containing 0.1 per cent 2:4 dinitrophenol for two days and 0.25 per cent 2:4 dinitrophenol for two days.

PLATE II

FIG. 1. Posterior portion of a lens from a chick receiving a diet containing 0.25 per cent 2:4 dinitrophenol for four days.

FIG. 2. Posterior portion of a lens from a chick receiving a diet containing 0.25 per cent 2:4 dinitrophenol for eight days.

FIG. 3. Posterior portion of a lens from a chick receiving a diet containing 0.25 per cent 2:4 dinitrophenol for eight days, plus riboflavin 0.35 mgm. per 100 grams intramuscularly every two days.

FIG. 4. Posterior portion of a lens from a chick receiving a diet containing 0.25 per cent 2:4 dinitrophenol for three days and 0.1 per cent 2:4 dinitrophenol for seven days.

FIG. 5. Posterior portion of a lens from a chick receiving a diet containing 0.15 per cent 2:4 dinitrophenol for fifteen days and 0.25 per cent 2:4 dinitrophenol for three days.

PLATE III

FIG. 1. Posterior portion of a lens from a chick receiving a diet containing 0.15 per cent 2:4 dinitrophenol for fifteen days and 0.25 per cent 2:4 dinitrophenol for seven days.

FIG. 2. Posterior portion of a lens from a chick receiving a diet containing 0.25 per cent 2:4 dinitrophenol for eighteen days and 0.12 per cent 2:4 dinitrophenol for twelve days.

FIG. 3. Anterior portion of a lens from a duck receiving a diet containing 0.25 per cent 2:4 dinitrophenol for one day.

FIG. 4. Posterior portion of a lens from a duck receiving a diet containing 0.25 per cent 2:4 dinitrophenol for one day.
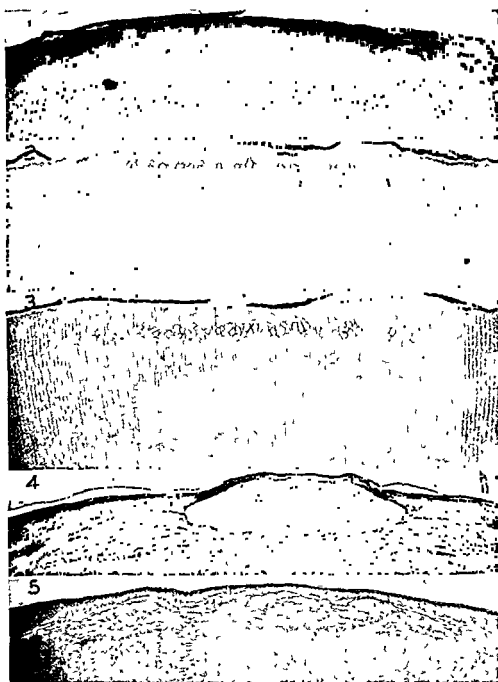
PLATE I
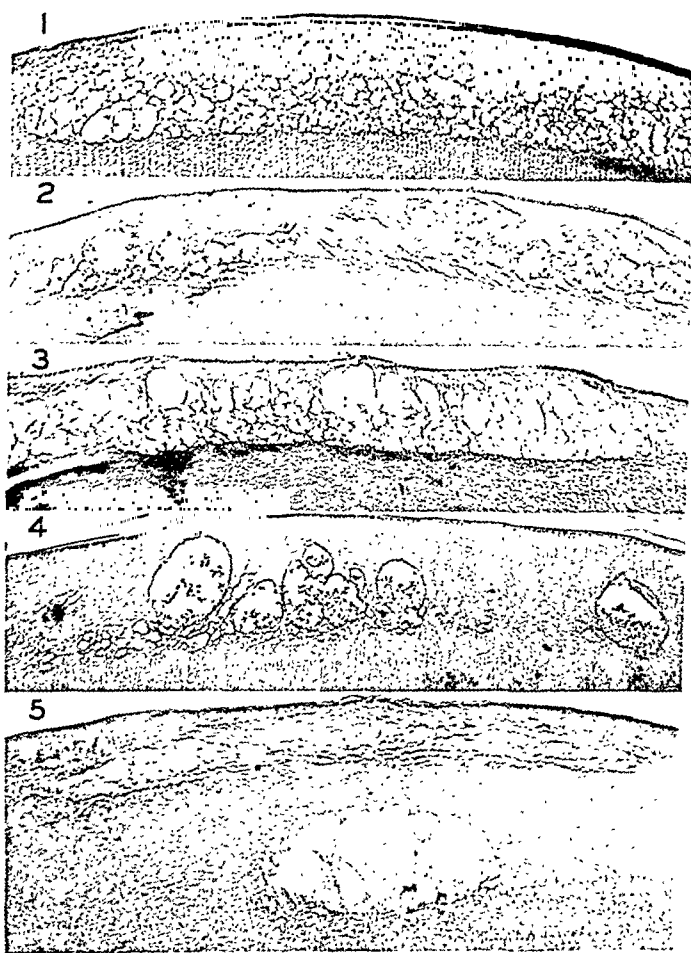
PLATE II
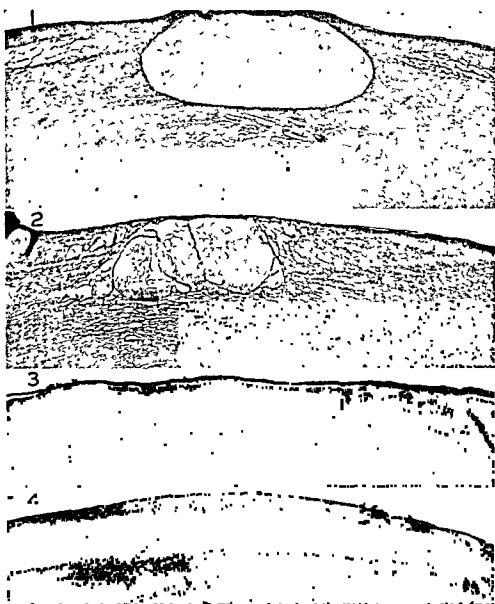
PLATE III

STUDIES ON VERATRUM ALKALOIDS

V. The Effect of Veratridine and Cevine upon the Circulation in Anesthetized Dogs, with Particular Reference to Femoral Arterial Flow[1]

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The depressor action of veratrum was already known when von Bezold and Hirt (1) described the effect of veratrine acetate. Since the publication in 1867 of their important study, the blood pressure decreasing action of the alkaloids of various species of veratrum has been the subject of numerous investigations. Pilcher and Sollmann (2) believed that the depressor action of Veratrum viride and cevadine in dogs was the result of cardiac inhibition, and that cevadine tended to produce "stimulation" of the vasomotor center as evidenced by slight vasoconstriction in the perfused innervated spleen, even in the presence of a decreased arterial pressure. Cramer (3), however, finding that the fall in blood pressure produced in cats by injections of Veratrum viride preparations was often unaccompanied by bradycardia but was prevented by vagal section, concluded that the hypotension was a result of the action of afferent impulses from the lungs upon the vascular reflex centers. MacNider (4) found that atropine did not prevent the fall in pressure in dogs caused by Veratrum viride and believed that vasodilatation was an important factor.

Jarisch and Richter (5), studying veratrine hydrochloride-Merck, found that the blood pressure fall in cats was not abolished by atropine and demonstrated that liver volume increased as blood pressure fell, which, in the absence of any increase in venous pressure, was interpreted as evidence of hepatic vasodilatation. They concluded from their experiments that the vasodilatation was due to vascular reflexes and that afferent sources of the reflex lay in the heart and also in the lungs (6).

Observations made by Krayer, Wood, and Montes (7) during the course of experiments on the sites of action involved in veratridine bradycardia revealed that when a dose of veratridine or veratrine was injected into and confined to the heart-lung circuit of a heart-lung-perfused head preparation, vasodilatation occurred in the head circuit, as evidenced by a transitory decrease in perfusion pressure (see figure 4, part A, of their paper). The vasodilator response in this case was evidently a reflex originating in certain chemosensitive endings in the heart or lungs and was mediated by afferent fibers in the vagi.

Two conclusions can be drawn from these brief references: (1) The majority of the authors quoted appear justified in assuming that vasodilatation plays an im-

portant role in the hypotensive effect of the veratrum alkaloids   (2) The hypo tensive action is due, at least in part, to a vascular reflex   In the experiments reported here, we have studied the problem in greater detail because in no pre vious investigation have direct measurements been made in intact animals of changes in arterial blood flow under the influence of any one of the veratrum alkaloids   We have used the alkamine cevine, and its veratric acid ester, veratri dine   The veratridine was prepared by Linstead and Todd (8), and the cevine was supplied to us by Dr Walter A Jacobs, who generously put at our disposal the following information about his sample of cevine   The elementary analysis yielded C 63 21, H 8 59, (calculated for cevine $C_{27}H_{43}O_8N$ C 63 61, H 8 51) The substance melted, not sharply, to a resin at 170–175°, depending somewhat on the rate of heating   No rotation was taken

METHODS   Experiments were carried out on dogs of both sexes weighing from 15 0 to 19 2 kgm and anesthetized with sodium pentobarbital 35 mgm /kgm intraperitoneally Six preliminary experiments were performed with the thermoelectric flowmeter units de scribed by Bennett Sweet and Bassett (9) and twenty three with differential manometers [adapted from the method of Fleisch (10)] attached to cannulas in the two femoral arteries Each differential manometer unit consisted of a U tube containing dyed chloroform under saline and was arranged to indicate the pressure gradient between two side tubes in a metal cannula the ends of which were tied into the proximal and distal stumps of the femoral artery   For a distance of one centimeter between the side tubes the diameter of the lumen of the cannula was 1 65 mm   at each end of the cannula the bore was about 2 5 mm   The pressure gradient across the cannula at a blood flow of 40 cc per minute (approximate aver age initial flow in the innervated femoral artery in these experiments) and with a relative blood viscosity of 5 was less than 2 mm of mercury   With the chloroform water system this represents an excursion of 50 mm   The relation between flow and pressure gradient at moderate flows closely follows the straight line predicted by the Poiseuille equation   At high flows the calibration deviates slightly equal flow increments incur greater pressure increments   Under conditions of high volume flow, when the pressure gradient is corre spondingly great the importance of collateral circulation increases   Consequently the *actual* flow through the vascular area normally supplied by the femoral artery must be some what greater than the recorded values and the higher the recorded flow the greater this error becomes (11)   Fortunately this means that the recording technique minimizes rather than exaggerates the actual extent of the vasodilatation produced by the drugs used in this study   Kymographic tracings of the pressure gradient were obtained manually by follow ing the chloroform water meniscus with a sliding indicator arranged to move a pointer on the smoked paper   At the high flows often encountered in the denervated leg a bromoform saline manometer was used with a sensitivity approximately one fourth that of the chloro form system   Estimations of relative blood viscosity were made at the beginning and end of three experiments   No significant change occurred

Arterial pressure was recorded from the axillary or carotid artery by means of a glass membrane manometer in the experiments with the thermoelectric flowmeter and by means of a mercury manometer in the others   In each case the manometer system was partially damped to permit estimation of the mean pressure   Respiration was recorded in most experiments with a tambour attached to the tracheal cannula   Heart rate was counted at 5 or 10 second intervals during the acute effects of drug administration   Right atrial pres sure was recorded optically in the preliminary experiments

In thirteen of the experiments in which blood flow in the two femoral arteries was re corded simultaneously one leg was denervated by section of the femoral obturator, and sciatic nerves and the sheath of the femoral vessels high in the thigh

In four experiments the left femoral artery of one dog (recipient) was perfused from the

left carotid artery of a second dog; blood from the femoral vein was returned to the external jugular of the donor; collateral circulation was interrupted by section of the muscles and skin high in the thigh, or by compression with an ecraseur. The femoral nerve, sciatic nerve, and femur remained as the only connection between the perfused leg and the recipient animal. Blood flow was recorded in both the intact and the perfused femoral arteries of the recipient dog.

Heparin[3] was administered in an initial dose of 2 mgm./kgm. intravenously, followed by continuous infusion of a 0.2% solution in physiological saline at a rate of 0.2 cc. per minute. Injections of drugs were made into the right atrium through a tube passed down the right external jugular vein; in six experiments veratridine was administered directly into the femoral artery through a 25-gauge needle. Doses of veratridine and cevine are given in terms of the base; both substances were injected in the form of the hydrochloride salt. Since the animals were selected within a narrow body-weight range, doses are stated as the *total dose rather than the amount per kilogram body-weight. Average dose per kilogram* is approximately 6% of the total dose.

RESULTS. VERATRIDINE. Veratridine in doses of 0.03 to 0.1 mgm. injected into the right atrium produces a rapid decrease in mean arterial pressure which reaches a minimum in about 30 seconds and returns to normal or to a plateau somewhat below normal in two or three minutes (fig. 1). The decrease in pressure is usually accompanied by a decrease in heart rate, and cardiac arrest for several seconds frequently occurs after an initial dose of 0.1 mgm. [In one experiment of this series no significant changes in heart rate were observed with doses up to 0.09 mgm. (see figure 2).] Accompanying the hypotension and bradycardia there is a vasodilatation in the vascular bed supplied by the innervated femoral artery. Following the injection of moderate doses which do not cause cardiac arrest the dilatation is great enough to cause an actual increase in the femoral volume flow (fig. 1; fig. 2), but if bradycardia in the initial phase of the drug action is severe, arterial pressure drops to a very low level and the femoral flow in such cases decreases, followed by an increase. In all cases the ratio of pressure to flow, as an approximate estimate of resistance, decreases, usually reaching a minimum value at the time of the maximum blood pressure response. As the blood pressure begins to rise, flow in the innervated artery decreases, often to a level below that preceding the injection, and may remain low for several minutes after the blood pressure has returned to normal; the ratio of pressure to flow is of course elevated during this phase. This suggests the intervention of pressor reflexes brought into action by the period of hypotension.

Small doses of the alkaloid may produce significant effects upon the circulation without any respiratory action, or occasionally with a slight acceleration of respiratory rate (fig. 2A). Larger doses invariably produce a cessation of respiration for a period of time proportional to the dose and lasting in some instances as long as 40 seconds (fig. 1). Doses causing apnea result in a moderate increase in apparent venous pressure as recorded in the right atrium. If apnea is prevented by artificial respiration, venous pressure remains unchanged. Artificial respiration, used in four experiments, did not significantly modify the depressor effect of veratridine.

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[3] Heparin-Connaught was used in most experiments; in the others, "Liquaemin," generously supplied by Hoffmann-La Roche, Inc., was used.

Dosage range In nine experiments veratridine was administered in progressively increasing doses in order to determine the minimal effective dose and the effect of repeated doses In animals under nembutal anesthesia and without previous morphine medication, no response was ever observed following a dose of 0 01 mgm , even when this was repeated every 5 or 10 minutes for a total of six injections A dose of 0 02 mgm produced a brief depressor effect in more than half the cases, and 0 03 mgm was always effective Figure 3 illustrates the relation of blood pressure response to dosage The experiments from which
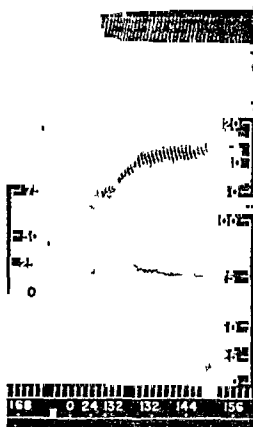
FIG 1 EFFECT OF VERATRIDINE ON BLOOD PRESSURE AND FEMORAL ARTERIAL FLOW
Experiment 17 Dog weight 15 0 kgm female Innervation of both legs intact Tracings from top to bottom respiration carotid arterial pressure (scale at upper right in mm Hg) blood flow in left femoral artery (scale at left in cc per min) blood flow in right femoral artery (scale at lower right in cc per min) time in 5 sec intervals At signal 0 05 mgm veratridine was injected into the right auricle Figures indicate heart rate per minute counts were actually made for 5 or 10 second intervals

this curve was constructed were all conducted in the same manner, namely by giving progressively increasing doses until a maximum response was reached Although repeated equal doses lead to roughly equivalent responses, 0 03 to 0 1 mgm given as an *initial* dose causes a somewhat greater response than would be predicted from the curve of figure 3 This effect is similar to, but much less pronounced than, the action of Veratrum viride preparations, which characteristically lead to marked tachyphylaxis (3)

The reflex nature of femoral vasodilatation In order to determine whether
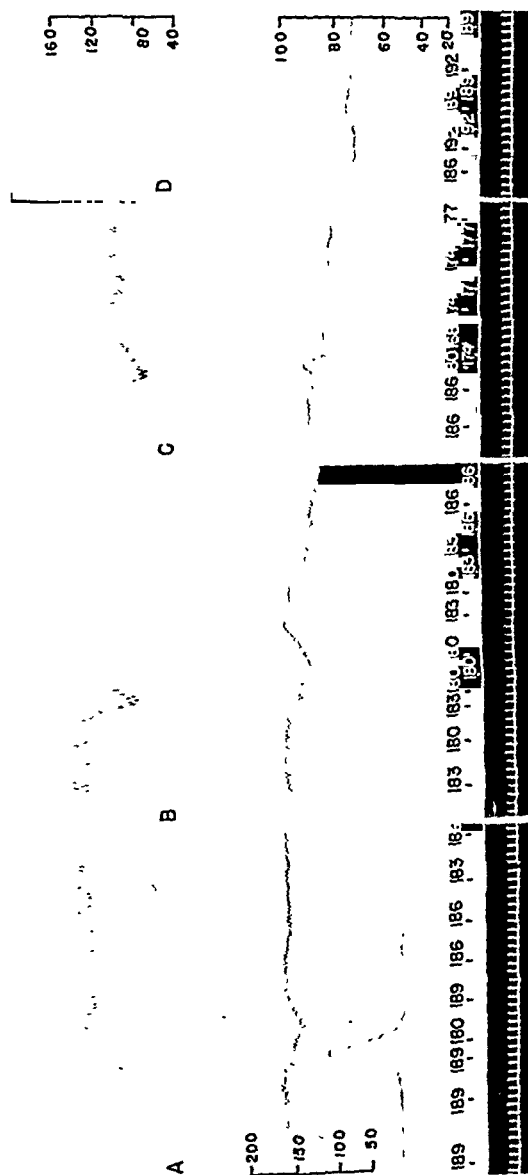
FIG. 2 EFFECT OF VERATRIDINE IN INCREASING DOSES UPON BLOOD PRESSURE AND FLOW; EFFECT OF VAGOTOMY

Experiment 6. Dog weight, 18.8 kgm., male. Left leg denervated at beginning of experiment. Tracings as in figure 1. Before part A, five injections of 0.01 mgm. and 3 of 0.02 mgm. were given without effect. At signal in segment A, veratridine 0.01 mgm. was injected into the right auricle. Between A and B, time lapse of 16 minutes; one intervening dose of 0.01 mgm. with effect comparable to A. At signal in B, veratridine 0.06 mgm. Between B and C, time lapse of 5 min. At signal in C, veratridine 0.09 mgm. Between C and D, time lapse of 16 min.; one intervening dose of 0.135 mgm., followed by vagotomy 3 minutes before signal of D. At signal, veratridine 0.135 mgm.

the dilatation of the femoral arterial bed was direct or *reflex*, veratridine was injected intra-arterially in six experiments. The doses used ranged from 0 01 to 0.5 mgm. in a total volume of 0.1 cc. and were injected within two seconds. Concentrations in the blood (up to 1:3,000) were higher than those reached by the largest doses given by vein. No significant change in blood flow occurred in any case; the vasodilatation occurring after intravenous injection is therefore not due to direct peripheral action. Further to test this and to provide a basis for comparison of the response of the innervated artery, the left leg was denervated by high section of the femoral, obturator, and sciatic nerves in thirteen
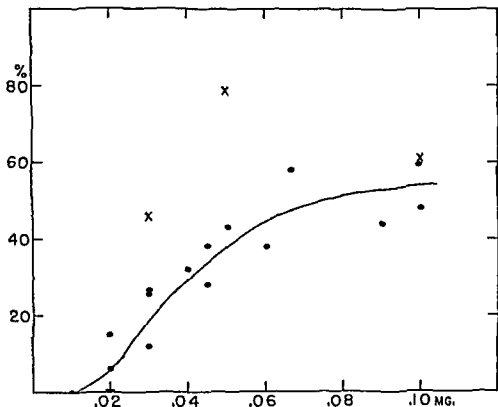
Fig 3 RELATION OF BLOOD PRESSURE RESPONSE TO DOSAGE OF VERATRIDINE
Ordinate, percentage decrease in blood pressure   Solid circles represent individual
                                                   is given in repeated doses beginning
                                                   in left to right the average responses
                                                   to 0 05 mgm in one experiment, and
to 0 1 mgm in 9 experiments

experiments. This procedure was performed usually before any veratridine had been given, but in three experiments denervation was performed between the administration of two equal doses of veratridine Denervation was always followed by a great increase in flow, reaching three or four times the flow on the innervated side. In several experiments blood flow in the denervated leg followed passively the systemic pressure after veratridine injection, but this does not prove the neurogenic nature of the vasodilator response to the drug for reasons which will be considered in the discussion.

The femoral artery is abundantly supplied with collateral vessels which

under certain conditions may contribute greatly to the inflow of this vascular net (11). In order to study the flow in the femoral artery free from the influence of collateral channels, an ecraseur was tightly applied to the thigh in one experiment, excluding the femoral vessels and the sciatic and femoral nerves. The dilator responses to equal doses of veratridine before and after application of the ecraseur were not significantly different. In four additional experiments the ecraseur was applied after perfusion of the femoral bed from a donor animal

FIG. 4. THE REFLEX NATURE OF THE VASODILATOR RESPONSE TO VERATRIDINE

Experiment 23, cross-circulation. Recipient dog weight 15.0 kgm., female; donor dog, 14.6 kgm., female. Tracings, top to bottom: respiration of recipient dog; carotid arterial pressure of donor dog (scale at upper left in mm. Hg); carotid arterial pressure of recipient dog (scale, upper right); blood flow in left femoral artery of recipient dog, perfused from donor (scale at left in cc. per min.); blood flow in right (intact) femoral artery of recipient (scale at right); time in 5 sec.; signal marker. Heart rate figures in beats per minute, recipient dog. At signal of A, veratridine 0.03 mgm. into right auricle of recipient dog. Between A and B, time lapse of 31 minutes; bilateral vagotomy of both animals. At signal in B, veratridine 1.0 mgm. injected into recipient dog.

had been established. Veratridine in doses of 0.02 to 0.08 mgm. injected into the recipient animal caused the typical dilatation response in the perfused leg, proving that this response is dependent upon nervous, not vascular, connections with the rest of the animal (fig. 4A). Similar doses of veratridine injected into the *donor* animal resulted in a fall in arterial pressure and consequently a passive *decrease* in flow in the perfused leg. The effects of larger doses in the cross-circulation experiments will be considered with the effect of vagotomy.

Effect of atropine From the experiments of Krayer, Wood, and Montes (7) it is evident that veratridine bradycardia can be abolished by vagotomy They showed that afferent sources of the reflex exist in the chest and probably in the carotid bodies, and that a central action is also present Experiments were therefore conducted to determine the role played by the vagus in the vasodilator response to veratridine

Atropine was administered in full doses (up to 12 mgm) in three experiments This would be expected to eliminate bradycardia as a factor in the blood pressure fall Following atropine administration the heart rate response to veratridine was largely, though not always completely, abolished In every case, however,

TABLE 1

Effect of vagotomy on the circulatory and respiratory responses to veratridine

EXP NO	BODY WT	SEX	DOSE	PERCENTAGE RESPONSE BEFORE AND AFTER VAGOTOMY											
				Arterial pressure			Heart rate			Femoral resistance*			Respiratory rate		
				B†	A	A/B	B	A	A/B	B	A	A/B	B	A	A/B
	kgm		*mgm*												
13	18 8	F	0 067	69	26	*38*	73	0	*0*	67	57	*85*			
15	15 9	M	0 067	68	25	*37*	76	3	*4*	67	37	*55*	93	0	*0*
16	15 3	F	0 08	47	28	*60*	48	6	*12*	70	71	*102*	92	0	*0*
3	16 5	M	0 10	77	39	*51*	93	4	*4*	64	67	*105*			
5	15 4	M	0 10	48	10	*21*	61	0	*0*	48	10	*21*	86	0	*0*
8	15 7	M	0 10	59	19	*32*	70	0	*0*	78	59	*76*	93	50	*54*
6	18 8	M	0 135	50	19	*38*	19	0	*0*	71	42	*59*	66	37	*56*
9‡	16 7	M	0 025	56	13	*23*	58	8	*14*	45	12	*27*	87	0	*0*
4‡	17 0	M	0 04	91	28	*31*	93	0	*0*						
7‡	15 3	F	0 04	69	38	*55*	93	10	*11*	63	64	*102*	84	0	*0*
Average						*39*			*5*			*70*			*16*

* Ratio pressure to flow in the innervated leg

† B = percentage decrease in pressure, heart rate, resistance, and respiratory rate *before* vagotomy, A = percentage decrease in response to same dose of veratridine *after* vagotomy, A/B = ratio of response *after* vagotomy to response *before* vagotomy, x 100

‡ Atropine sulfate administered with the anesthetic

the blood pressure fell, though not as low as in control observations This confirms the observation of MacNider (4) and of Jarisch and Richter (5), who concluded that bradycardia was not the sole cause of the depressor response to veratrine Atropine had no effect upon the respiratory response

Effect of vagotomy Vagotomy, which interrupts both efferent cardio-decelerator pathways and afferent fibers from the receptor areas in the chest, in almost all cases *decreased* the depressor response, but rarely abolished it Table 1 represents the pertinent results of eleven experiments in which vagotomy was performed In experiments 3, 7, and 16 the dilatation in the innervated femoral artery was as great after vagotomy as with equal doses before vagotomy In nine experiments the effect of veratridine upon heart rate and respiratory rate

under certain conditions may contribute greatly to the inflow of this vascular net (11). In order to study the flow in the femoral artery free from the influence of collateral channels, an ecraseur was tightly applied to the thigh in one experiment, excluding the femoral vessels and the sciatic and femoral nerves. The dilator responses to equal doses of veratridine before and after application of the ecraseur were not significantly different. In four additional experiments the ecraseur was applied after perfusion of the femoral bed from a donor animal

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had been established. Veratridine in doses of 0.02 to 0 08 mgm. injected into the recipient animal caused the typical dilatation response in the perfused leg, proving that this response is dependent upon nervous, not vascular, connections with the rest of the animal (fig. 4A). Similar doses of veratridine injected into the *donor* animal resulted in a fall in arterial pressure and consequently a passive *decrease* in flow in the perfused leg. The effects of larger doses in the cross-circulation experiments will be considered with the effect of vagotomy.

FIG 5 EFFECT OF VAGOTOMY ON THE VASCULAR RESPONSE TO VERATRIDINE

Experiment 13   Dog weight 18 8 kgm , female   Tracings as in figure 1   Left leg denervated at start of experiment
Injection of morphine, 20 mgm per kgm , preceded segment A   At signal of A, veratridine 0 067 mgm into right auricle
Between A and B, time lapse of 10 minutes and bilateral vagotomy At signal of B, veratridine 0 067 mgm   Between B
and C, time lapse of 27 minutes   At signal of C, veratridine 10 mgm   Heart rate figures, beats per minute, but counted
for 5 or 10 second intervals

Characteristic results are illustrated in figures 4B and 5C. As mentioned above, doses of the order of 0.1 mgm. still resulted in a decrease in blood pressure. With progressively larger doses, however, the depressor response eventually disappeared, and doses of from 0.5 to 1.0 mgm. always caused a significant increase in carotid pressure, reaching in one case 250 mm. mercury. The pressure began to rise 13 to 26 seconds after the veratridine injection, reaching a maximum in 1 to $3\frac{1}{2}$ minutes and gradually returning to normal in 6 to 12 minutes. Coincident with the ascending limb of the blood pressure tracing there was an increase in flow in both innervated and denervated femoral arteries, followed by a decrease at the height of the blood pressure response; but the ratio of pressure to flow was always increased, in some instances more than doubled, suggesting actual constriction. Since this occurred also in the denervated limb it is probable that epinephrine is at least in part responsible for the hypertension. The heart rate was always increased as the pressure rose, rising in one case to 252 per minute from an initial rate of 162. Respiratory effects were inconstant with these doses; frequently a period of apnea occurred, probably as a result of a carotid sinus reflex.

The cross-circulation experiments afforded an opportunity to investigate further the nature of the hypertensive response to large doses. Bilateral vagotomy was performed in both donor and recipient dogs in three experiments. Veratridine in a dose of 1.0 mgm. injected into the recipient animal evoked the characteristic epinephrine-like flow response of the intact leg, accompanied by a long-lasting dilatation in the perfused leg. That is, there was active constriction in the intact leg, and dilatation in the leg which was connected only by nervous pathways to the recipient animal (fig. 4B). It is probable, therefore, that the large dose of veratridine still produced the dilator response, but that this was masked by the simultaneous action of epinephrine in the intact leg. Epinephrine, of course, could not reach the perfused leg. Conversely, veratridine in doses of 1.0 mgm. injected into the donor animal (connected only by vascular channels with the perfused leg) caused an increase in donor arterial pressure with an initial flow increase in the perfused limb. The flow rapidly decreased, however, as the pressure reached its maximum. This response is not due to a peripheral constrictor action of the large dose of veratridine (veratridine in doses as high as 0.5 mgm. intra-arterially failed to cause any constriction), but must be ascribed to epinephrine released from the adrenals of the donor dog and reaching the perfused leg of the recipient dog in high enough concentration to cause active constriction.

2. CEVINE. Cevine was studied in two experiments. Doses up to 40 mgm. were without effect upon arterial pressure or femoral flow in one experiment in which a good response was obtained with 0.02 mgm. of veratridine. In the second experiment a questionable femoral dilatation was obtained with 40 mgm. as an initial dose; and a dose of 80 mgm. doubled the blood flow in the innervated leg. This dilatation was unaccompanied by any change in heart rate, respiratory rate, or blood pressure. It is evident that cevine lacks the characteristic depressor property of veratridine even in doses up to 2000 times

the minimum effective dose of the ester alkaloid This is in accord with previous investigations which have already emphasized the qualitative difference between the effect of cevine and of veratridine in the cat (12), as well as in the dog (7)

Discussion The results presented above demonstrate that veratridine produces an abrupt fall in blood pressure as a consequence of reflex bradycardia and vasodilatation, section of the vagi in the neck eliminates the bradycardia and reduces but does not abolish the vasodilatation That the latter effect is neurogenic and not the result of a direct action of the alkaloid upon arterial smooth muscle is evident from the response of the perfused limb in the cross circulation experiments, as well as from the lack of response to veratridine when injected intra arterially Denervation of the leg, which alters the flow response of the femoral bed, does not give clear cut proof of the reflex nature of the dilatation, since the capacity of the denervated vascular bed to respond to a vasodilating agent is doubtless greatly reduced The importance of collateral vessels retaining their nerve supply, in modifying the expected passive response of the femoral bed in the acutely denervated leg is obvious

The period of apnea resulting from moderate doses of veratridine may well modify the circulatory response In two experiments we have observed well marked dilatation and fall in blood pressure without any respiratory inhibition In others artificial respiration did not abolish the vascular effect Moreover, while the chemical effect of a period of apnea would be expected to produce vasodilatation, the reflex cycle initiated by anoxic stimulation of the carotid bodies would lead to the mobilization of pressor mechanisms In one experiment veratridine in a dose of 0 03 mgm caused a greater percentage fall of blood pressure and of femoral resistance during a period of artificial respiration than equal doses of the alkaloid before and after this period Furthermore vagotomy, which largely abolishes the respiratory response to small doses, frequently had only a minor effect upon the vasodilator response (table 1)

Morphine sulfate, which was injected in a dose of 2 mgm per kgm in four experiments, appeared to enhance the depressor response to veratridine (see tables 1 and 2) Further experiments are necessary to establish the validity of this observation

It was pointed out that intra arterial injections of veratridine leading to concentrations up to 1 3 000 had no effect upon blood flow This is peculiar in view of the fact that vasoconstriction was observed by various authors when veratrine in concentrations between 1 100 000 and 1 1 000 was perfused through isolated organs (13 14) It is possible that in our experiments the period of action of a single dose was too brief to result in constriction

It is difficult to draw any comparison between the present work, which was carried out with pure alkaloids and previous studies on the circulatory action of such mixtures as veratrine and tinctures or fluid extracts of Veratrum viride and Veratrum album One of the active principles of the latter preparations, protoveratrine differs in many respects from veratridine, as will be shown in later communications In contrast to the effects of Veratrum mixtures, veratridine can be injected repeatedly with little tachyphylaxis

Cevine, which caused a significant dilatation of the femoral bed when injected in large doses, is known to exert a convulsant action upon the central nervous system (12). It is possible that the dilatation observed after a dose of 80 mgm., which was not accompanied by a fall of blood pressure, was the result of an increase in muscular activity of the leg, although no gross movements were noted.

SUMMARY

1. Veratridine injected intravenously in dogs of 15 to 19.2 kgm. body weight in doses of from 0.02 to 0.14 mgm. produces an abrupt fall in blood pressure and heart rate, and respiratory inhibition, by way of reflexes mediated partly but not solely by afferent fibers in the vagus nerves.

2. The fall in blood pressure results partly from bradycardia and partly from a vasodilatation of the arterial tree as represented by the femoral arteries.

3. The peripheral dilatation is neurogenic and not the result of a direct action upon vascular smooth muscle. It is independent of the respiratory action.

4. Vagotomy almost completely eliminates the heart rate and respiratory effect of small doses of veratridine, and reduces, but does not abolish, the vasodilatation.

5. Large doses of veratridine (0.5 to 1.0 mgm.) cause, after vagotomy, an increase in blood pressure and heart rate, probably partly as a result of epinephrine liberation. The action of the epinephrine on the peripheral vessels masks a simultaneous decrease of vasoconstrictor tone.

6. Cevine lacks the characteristic depressor effect of veratridine in doses up to 2000 times the minimal effective dose of its veratric acid ester.

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CHOLINE ESTERS WITH ATROPINE-LIKE ACTION[1]

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A peripheral atropine like action of certain choline esters which were recently synthesized by the authors (1) is reported herein

The pharmacologic action of choline esters has been considered to have two main components (2) The stimulatory action on the effector organs of the cholinergic nerves of salts of choline, acetylcholine, acetyl beta methyl choline, and carbamylcholine resembles that of muscarine The muscarine like effects of cholines are effectively blocked by atropine and other members of the belladonna series Many choline esters, notably carbamylcholine and acetyl choline, also have a nicotine like action, i e, a stimulatory effect on ganglionic synapses of the sympathetic and parasympathetic nervous system and on skeletal muscle These effects are inhibited by curare and large doses of nico tine and atropine

Choline esters with muscarine- and nicotine like actions are relatively small molecules containing a quaternary ammonium group As this group is highly polar, salts of the choline esters such as acetylcholine and carbamylcholine have the physical properties of polar compounds, i e, they have a high affinity for water but are practically insoluble in organic solvents and tissue lipids They are surface inactive, e g, they have little influence on the surface tension of water It was conceived that choline compounds with modified physical proper ties might have altered pharmacological effects Development of surface active derivatives was considered to have greatest promise for it was known that surface activity had a profound influence on biologic phenomena, e g, on the activity of narcotic agents and on cellular permeability (3)

Surface activity of organic compounds is largely dependent on the presence of polar and non polar groups at opposite ends of the molecule Large mole cules with this structure orient themselves at the surface of water and thereby lower the air water interfacial tension To make choline derivatives with this structure necessitated the placement of the large non polar group at the opposite end of the molecule from the highly polar quaternary ammonium group, i e, esterification of choline with acids containing large non polar groups The most stable and potent of known choline esters, carbamylcholine, was selected for modification The nitrogen atom in the carbamyl group has two replaceable hydrogens replacement of each hydrogen by a chain of four or more carbons, or an aromatic ring makes the carbamyl end of the molecule predominantly non polar, e g, di n butyl carbamylcholine chloride (fig 1) Five % aqueous

solutions of di-n-butyl-carbamylcholine chloride reduce the air-water interfacial tension to less than 45 dynes per cm. at 25°C. Like other surface-active agents, it has detergent and bactericidal properties. Concentrations of 0.2–0.5% promptly sterilize 24 hour broth cultures of hemolytic staphylococci and streptococci.

In the mammalian eye instillations into the conjunctival sac of 5–10% solutions of di-n-butyl-carbamylcholine chloride produce mydriasis and cycloplegia as compared to miosis and cycyltonia produced by carbamylcholine. Even when the new drug is injected[2] into the anterior chamber in threshold concentrations (0.0001%) mydriasis is not preceded by stimulation of the iris sphincter, i.e., miosis. In contrast, carbamylcholine produces miosis even when injected in lethal doses. The ocular effects of the new drug, therefore, resemble those of atropine rather than the muscarine-like action of previously known choline esters. In puppy eyes enucleated while under the influence of di-n-butyl-carbamylcholine and studied histologically, the morphology of the ciliary body is similar to that seen in paresis of the ciliary muscles produced by excision of the ciliary ganglion. In the human eye, there is recession of the near point of accommodation similar to that produced by atropine.

$$(CH_3)_3 \cdot \underset{Cl}{N} \cdot (CH_2)_2 \cdot O \cdot \overset{O}{\underset{}{C}} \cdot N \cdot \mathbf{H_2}$$ CARBAMYLCHOLINE CHLORIDE (DORYL)

$$(CH_3)_3 \cdot \underset{Cl}{N} \cdot (CH_2)_2 \cdot O \cdot \overset{O}{\underset{}{C}} \cdot N \cdot \mathbf{(C_4H_9)_2}$$ DI-n-BUTYL-CARBAM-YLCHOLINE CHLORIDE
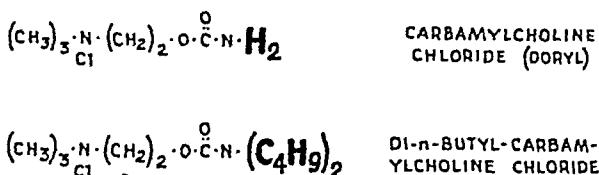
FIG. 1

The new drugs, like atropine, produce mydriasis by paresis of the iris sphincter rather than by stimulation of the dilator fibers. In rabbit eyes under the influence of di-n-butyl-carbamylcholine, reactivity of the sphincter to electrical stimulation of the oculo-motor nerve is greatly reduced while reactivity of dilator fibers to stimulation of the cervical sympathetic nerve is not significantly effected. In humans mydriasis produced by di-n-butyl-carbamylcholine is submaximal, but becomes maximal when the dilator is stimulated, e.g., when epinephrine is administered. Müller's palpebral muscle, another smooth muscle with adrenergic endings, also is unaffected by di-n-butyl-carbamylcholine. There is no widening of the palpebral fissure as is produced by instillations of cocaine and epinephrine into the conjunctival sac.

Alone,[3] the new drug does not block nerve impulse to skeletal muscle. In

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[2] A double syringe system was devised so that the fluid contents of the anterior chamber could be exchanged without alteration of the intraocular pressure or the anatomical relationships of the iris. A single instillation of 1% tetracaine hydrochloride provided superficial anesthesia adequate for corneal puncture.

[3] In combination with physostigmine, di-n-butyl-carbamylcholine produces paralysis of skeletal muscles in rabbits. This unique synergism will be described in detail in a subsequent paper.

rabbits under ether or nembutal anesthesia, the superior rectus muscle was exposed through a conjunctival incision. The muscle tendon was detached from the eyeball and hooked to a muscle lever. Contractibility of the muscle following electrical stimulation of the oculomotor nerve was only slightly reduced by direct applications of 0 1% di n butyl-carbamylcholine chloride to the muscle. In birds in which the iris muscles are of the skeletal type, the new drug does not produce mydriasis when instilled into the conjunctival sac in 10% solution

From the above experiments, it appears that di n butyl carbamylcholine like atropine produces depression of smooth muscle with cholinergic endings (mammalian iris sphincter and ciliary muscle) but has little if any influence on smooth muscle with adrenergic endings (iris dilator and Müller's muscle)

The above experiments do not establish the exact site or mechanism of action of the drugs but there is some indication that the new drugs and the atropine series differ in this respect. Homatropine and di n butyl carbamylcholine simultaneously injected into the anterior chamber of the rabbit eye produce dilatation of the pupil in doses approximately one fourth of the minimal effective dose of each drug injected separately. Therefore, the action of the two drugs is synergistic rather than purely additive as might be expected if the site and mechanism of action of the two drugs were the same. Furthermore, the similarity of action of di n butyl carbamylcholine and atropine is purely qualitative. The duration of action of di n butyl carbamylcholine is much shorter than that of atropine, or even homatropine. The mydriatic action of the atropine series is much more pronounced than their cycloplegic effects, e g , with small doses of homatropine it is possible to produce a wide dilatation of the pupil without cycloplegia in the human eye. With di n butyl carbamylcholine mydriasis and cycloplegia often develop simultaneously, but in many emmetropic young adults, recession of the near point of accommodation (0 50 print) to 30–50 cm precedes any dilatation of the pupil

As yet, the only clinical use of the new drugs has been in cycloplegic refraction and routine internal examination of the eye (4). For this purpose, di n butyl carbamylcholine has the advantage of short action and negligible systemic effects from ocular administration but requires larger doses than the atropine series and occasionally produces a mild transitory disturbance of the corneal epithelium. Small doses of homatropine and di n butyl carbamylcholine may prove more ideal because of their synergistic action than a large dose of either drug alone. Also the bactericidal and detergent actions of the new drugs may be advantageous in the treatment of ocular inflammations

DISCUSSION   The reversal of action of carbamylcholine by addition of non polar groups is unparalleled in autonomic pharmacology. Several autonomic drugs notably nicotine produce stimulation in small doses and depression in large, but the reversal of action of carbamylcholine is not a dosage phenomenon. Even in threshold concentrations in the aqueous humor, depression produced by di n butyl carbamylcholine is not preceded by stimulation as judged by size of the pupil, moreover, high concentrations of carbamylcholine in the anterior chamber do not produce primary depression of the iris sphincter

SUMMARY

Surface-active choline esters were prepared by balancing the highly polar quaternary ammonium group in carbamylcholine with non-polar groups at the carbamyl end of the molecule.   The new compounds, e.g., di-n-butyl-carbamyl-choline chloride, have an atropine-like action on the ocular muscles rather than the muscatine- and nicotine-like effects characterizing previously known choline esters.

Di-n-butyl-carbamylcholine has short action and a proportionately greater effect on accommodation than on the pupil while atropine has more prolonged action and a greater effect on the pupil.   The new drugs and atropine act synergistically on the iris sphincter.

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THE CHRONIC ORAL TOXICITY OF SELENIUM

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Several papers which suggest the possibility of selenium poisoning in humans have been published (1, 2, 3, 4)   These were reports of studies made on a number of families in the so-called selenium belt, mostly in South Dakota   The symptomatology of the individuals has not offered definite proof that selenium is the cause of the illness   Nevertheless, the discovery of the presence of selenium in amounts as high as one part per million in the urine of people living in seleniferous areas has appeared alarming   From a consideration of these reports we have been interested in the possibility that the small amount of selenium sometimes occurring in foods might produce toxic effects   It has been our primary purpose, therefore, to determine the chronic toxicity of selenium at low levels of intake

Investigators of the chronic effect of selenium are generally in fair agreement that 10 or more parts per million in the diet of the rat is toxic   This phase of the selenium problem has been discussed in the recent review of Moxon and Rhian (5)   To account for differences in results the great variation in individual susceptibility has been mentioned by Smith (6), and the effect of diets has been reported by several authors (6, 7, 8, 9, 10)   It is known, also, that young rats are more susceptible to selenium than adult rats (7) and that female rats are more susceptible than male rats (10, 11)   An analysis of the various factors involved in the discrepancies in reported chronic toxicity studies made it appear probable that there might be two stages in selenium toxicity   In an attempt to answer this question we have continued our chronic toxicity studies for the approximate lifetime of the rat

EXPERIMENTAL   Female rats of our inbred Osborne-Mendel strain were fed ten different rations beginning at three weeks of age   A principal ingredient of all rations was grain in the ratio of 49 parts of corn to 44 parts of wheat   In three diets normal corn was replaced in part by selenium bearing corn to give dosages of selenium of 5, 7, and 10 parts per million   In another three diets normal wheat was similarly replaced with selenium wheat to give the same dosages of 5, 7, and 10 parts of selenium per million   Three more diets contained grain mixed with a sufficient quantity of an inorganic selenide[2] to furnish 10, 20, and 40 parts per million of selenium   The tenth ration was the control diet without selenium

In order to insure a balanced distribution of litter mates, four female rats in each of fifteen litters were assigned to ten different diets as required for balanced incomplete blocks of four with ten treatments   This design was replicated as a whole three times, giving a total of 180 rats   The rats were kept in individual cages in an air conditioned room for the duration of the experiment

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Length of Survival. The most striking feature in an initial inspection of the data was the wide difference in the length of the survival period. Some rats died within a few weeks; others were living after two years when the experiment was terminated. Between these extremes the mortality rate depended primarily upon treatment but was not uniform over the interval. Instead the frequency distribution of length of survival tended to be U-shaped, especially when the selenium was given in grain. With the cumulative frequencies plotted on logarithmic-probability paper, the more detailed study of the mortality rate at the earlier periods indicated a break or plateau with relatively few deaths from eight weeks to one year. This tendency disappeared in the diets containing the two high concentrations of selenium from the selenide, but the discrepancy may have been due to the use of higher dosages than in grain. Based upon this distribution of survival time, the toxic action could be divided into a subacute effect

TABLE 1

Mortality of rats on selenium

	DOSAGE OF SELENIUM	AT 8 WEEKS	AT 1 YEAR
	p.p.m.	%	%
Corn	5	11	17
	7	33	39
	10	67	72
Wheat	5	0	5
	7	44	61
	10	67	72
Selenide	10	11	56
	20	72	100
	40	94	100
Control		0	11

occurring within eight weeks and a chronic effect which might be survived a year or more (table 1).

Analysis in terms of percentage kill. The distribution of the mortality rate showed a relatively stable condition after an interval of approximately eight weeks on the experimental diets with selenium-bearing grain. Accordingly the percentage of rats killed by each treatment in eight weeks was selected as a second possible criterion. These data were transformed to probits (12) and plotted against log-dose in figure 1. Computed as corrected probits and weights, the observations agreed with the fitted curves well within the experimental error. Selenium in wheat and in corn were equally toxic with the toxicity in wheat estimated at 99.3 ± 9.8 per cent of that in corn. Combining the data from corn and wheat, the toxicity of selenium in the selenide proved by a similar calculation to be 47.9 ± 5.1 per cent of that for selenium in grain. For the rats in this particular experiment the concentration of selenium (when contained in

corn or wheat) in their diet, killing one-half of them in eight weeks, was 8 33 ± 0 46 parts per million   When given as a selenide, the LD50 of selenium was 17 40 ± 1 57 parts per million

Growth curves during youth   To correspond to the analysis in terms of percentage killed during the first eight weeks a study was made of the effect of dosage over this same interval   The rats were three weeks old when placed upon the experimental diet but in order to simplify handling the rats, the examination at the end of the first week was advanced or retarded by several days in some cases, so that all individuals could be weighed on the same day of the week   Hence, the time between the first and second weighings varied in length from four to ten days and was omitted from later calculations, giving growth curves based upon the weights from the first to the eighth week inclusive   Many growth curves were plotted individually to study the general form of the curve over this
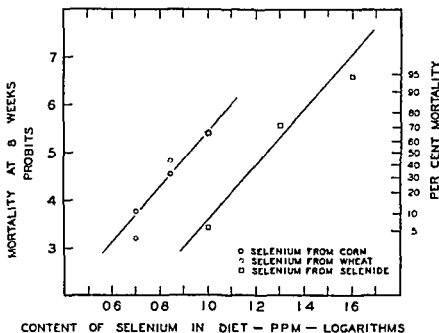
FIG 1 DOSAGE MORTALITY CURVE

period   The weight of the rat plotted against time in weeks approximated a straight line without significant curvature   By avoiding the first four weeks of growth, the logarithmic phase of the growth curve was missed in most cases, by terminating the curve at eleven weeks, the growth rate had not begun its decline as the rat approached maturity   Moreover, after approximately one week on the experimental diet, the rat presumably had become adjusted to its new regime and was eating more or less regularly

The mean growth rates were computed with the orthogonal polynomials for the linear term, omitting those measuring curvature   Where the record was complete, the curve was determined from the eight body weights for the first to the eighth week inclusive in the usual way   Many rats, however, died in less than eight weeks and for them the curve computed from the data for the first week to the last week of growth   In many cases the rats gained regularly up to

the last weighing before death, but frequently the rate of gain either dropped abruptly or reversed itself from one to several weeks before death. Since the present phase of the study concerned effects on growth apart from the symptoms of approaching death, the latter portion was omitted.

Since the addition of selenium to the diet divided the rats into two categories, a more susceptible group which died within eight weeks and a more resistant group which survived eight weeks or longer, the differences have been tested against the variation within categories of the growth rate of individual rats. The growth rates or regression coefficients, however, were not of equal reliability. The factor contributing most to this divergence—and the only one of importance here—was the unequal period covered by the different curves. For rats dying in less than eight weeks, the growth rate often had to be computed from the record of only two or three weeks, and in consequence had a smaller reliability

TABLE 2

Mean growth rate for first eight weeks on experiment

	DOSAGE OF SELENIUM	DIED WITHIN 8 WEEKS		LIVED MORE THAN 8 WEEKS		
		No. of curves	Mean growth	No. of curves	Mean growth	
	p.p.m.		*grams/week*		*grams/week*	
Corn	5	2	10.15 ±2.32	16	14.30 ±.49	1.278
	7	6	12.97 ±1.03	12	13.49 ±.56	.443
	10	11	9.84 ±1.24	6	14.08 ±.43	3.225
Wheat	5	0		18	14.43 ±.42	
	7	8	11.43 ±1.07	10	13.95 ±.99	1.727
	10	12	11.16 ± .60	6	13.71 ±.86	2.418
Selenide	10	0		16	13.99 ±.33	
	20	9	7.03 ±1.21	5	5.79 ±.81	—.845
	40	5	2.49 ±1.29	1	3.50	
Control		0		18	14.96 ±.60	

than if based upon the full series of eight weekly weighings. To compensate for this inequality a method suggested by R. A. Fisher for an analogous case has been adopted in computing mean growth rates and their standard error. Each growth rate was weighted by $\sqrt{S\,(x - \bar{x})^2}$ where x is age at any week and $\bar{x}$ is the mean of the weekly ages of the rat in question.

The weighted mean growth rates which have been computed for each treatment and category are listed in table 2. At the higher dosages, some rats lost weight from the beginning on the test diet and died within two or three weeks. Hence, there were fewer growth rates available with these treatments. On selenium-bearing grain, the growth rate tended to decrease as the dosage was increased within either group of rats, those living eight weeks or less and those living more than eight weeks, but the decrease was too small to be considered established with this number of individuals. Between groups, however, the more susceptible rats consistently grew at a slower rate on the selenium-bearing

gram, the difference being highly significant   If not separated on the basis of survival beyond eight weeks, the mean growth rate decreased steadily as the dosage increased but this was due primarily to the changing proportion of rats from the two categories

The higher dosage of selenium from the selenide reduced growth to a marked degree   The evidence indicates that if the animal ingested selenium over its tolerance, its growth rate before death was diminished by approximately twenty per cent   If the dosage was too small to kill the animal within eight weeks the selenium may still have retarded the rate of growth but the effect was too small and the growth rate too variable from one animal to another to consider it es tablished

Effect of selenium on late growth   At six months the growth rate settled down to a fairly even increase in weight approximately one-sixth of that during the

TABLE 3

Analysis of variance for average increase in rate during 26th to 50th week inclusive corrected by covariance for growth rate during first 8 weeks in terms of reduced mean squares

	D F	MEAN SQUARE	F
Effect of early growth	1	4 9118	6 98*
Variation between treatments in effect early growth	7	9315	1 32
Between 7 treatments and control	7	2 4804	3 53†
Corn vs wheat average 3 dose levels	3	1 3359	1 90
Between dose levels corn and wheat	2	6 1482	8 74†
In grain vs in selenide at 10 p p m	1	9288	1 32
High doses (5) vs control	1	1 7145	2 44
Low doses (2) vs control	1	2 5925	3 68
Error within doses pooled corrected for early growth	78	7035	1

* P < 05 > 01
† P < 01

first eight weeks   Growth curves for a number of individuals showed in general a linear trend in more than three fourths of the 30 cases that were plotted Hence, the average growth rate was computed with orthogonal polynomials for weight of rat against weeks over the interval from 26 to 50 weeks inclusive on the test diet   A few individuals died within this interval or shortly thereafter, but most of them lived much longer, so that the growth rates could be considered of equal reliability

The mature growth rates in grams per week were obtained from the 5 to 18 survivors on the eight different treatments   At this age the effects of litter differences presumably had disappeared and would be very difficult to evaluate in any case because of the many losses at an earlier age   Neither of the two higher dosages of the selenide was represented   The variance of the mature growth rate decreased with the number of survivors

The 87 observations on the mature growth rate were analyzed by covariance with the results summarized in table 3 in terms of the reduced mean squares

namely the formation of adenomas and low grade non-metastasizing hepatic cell carcinomas in 11 of 53 rats surviving at least 18 months. Four other rats showed marked adenomatoid hyperplasia, interpreted as a transitional stage in the formation of the tumors. In over 500 other rats of equal age, either controls or rats fed substances other than selenium, the incidence of such liver tumors has been less than one per cent. The tumors in the selenized rats were both single and multiple and as large as $3\frac{1}{2}$ cm. in diameter. They have already been described and illustrated (14) and as far as we know are the first reported tumors arising in experimentally cirrhotic livers after the cirrhosis has been present without tumor for a relatively long time.

Generally speaking, damage to the liver was of two types. Rats dying during the first three months, usually between the third and sixth weeks, showed a subacute type of damage with varying degrees of hepatic cell necrosis, atrophy, hyperplasia, cystic dilatation of sinusoids, and focal myelosis. Fibrosis, pigmentation, bile duct proliferation and intrahepatic hemorrhage were slight or absent. Gross abdominal hemorrhage was very frequent and appeared to originate from rupture of distended subcapsular sinusoids; often there was an organizing, pericapsular hemorrhagic exudate. After three months there was seen a chronic type of liver damage without intrahepatic hemorrhage or necrosis, with less of the cystic sinusoids and myeloid foci seen previously, and with increasing portal fibrosis, distortion of normal architecture, focal capsular retraction, hepatic cell atrophy and focal hepatic cell hyperplasia; in short, portal cirrhosis. Nodularity was sometimes so marked that the smaller liver lobes grossly resembled miniature bunches of grapes. Ascites was occasionally present, and hemoperitoneum of undetermined origin rarely present. Pigmentation of the liver, by hemosiderin or otherwise, was usually slight. In the period from two to five months, a blend of the two types of damage was seen.

In table 6 the detailed incidence of cirrhosis of the liver is given. One hundred of the 146 rats survived more than three months, and in 71 of these some degree of hepatic cirrhosis was present. The cirrhosis was graded as slight in 28 instances, moderate in 18, advanced in 21 and extreme in 4.

Experimental lesions caused by selenium in viscera other than the liver are rather variable and depend chiefly on the dosage, the species of animal used and the age of the animal; in our groups of rats none was characteristic or extensive. The basal diet is considered somewhat suboptimal in protein, and for this reason both control and test rats showed a slight fatty degeneration of the liver as well as a slight atrophy; in the test rats the latter was of course often lost in the more marked atrophy accompanying the cirrhotic process. The usual "spontaneous" pathological lesions in the control rats were slight, even at the end of two years. Using these as a base line, the selenized rats showed in general slight hyperplasia and hemosiderosis of the splenic pulp, slight hyperplasia of the bone marrow, slight focal myocardial fibrosis and minor renal changes. The hyperplasia in the bone marrow and spleen tended to be erythroid in the younger animals and myeloid in the older. Blood was rarely seen in the intestines. There were no pancreatic, pulmonary, adrenal or gross cutaneous alterations, and osseous

lesions were occasional and slight   In the younger rats there was slight Kupffer cell and splenic reticulum cell hyperplasia

TABLE 6
Incidence of cirrhosis of liver

DOSAGE SELENIUM	RATS IN GROUP	DIED FIRST 3 MO	CIRRHOSIS IN INDIVIDUAL RATS			
			3½–11½ mo	12–17½ mo	18–23½ mo	24 mo
3 p p m , in corn	19	0	+, +, +, +, +, +, ++, ++, +++	0, 0, 0, 0, 0, 0, 0, +, +, +++*		
5 p p m , in corn	18	2	0	0, 0, 0, ++, +++	+	0, 0, 0, 0, 0, +, +T, +T, ++
5 p p m , in wheat	18	0	0	?, 0, +	0, 0, 0, +	0, 0, +, +, +, +T, ++, ++, ++, ++T
7 p p m , in corn	18	7		+++	+, +, ++	+, +, +A, ++, +++T, +++T, ++++
7 p p m , in wheat	18	9	++, ++	0, ++, +++	+T	++, +++A, ++++
10 p p m , in selenide	18	2	?, 0, 0, +, ++, +++	0, +, +++	++++A	+, +++T, +++, +++, +++, +++T
10 p p m , in corn	18	13			++, +++A	++, +++T, +++T
10 p p m , in wheat	18	12	+	+++, +++	+++, +++	++++
Totals	145	45	20	27	14	33

    + = Slight
    ++ = Moderate
    +++ = Advanced
++++ = Extreme
    T = Tumor
    A = Adenomatoid hyperplasia
    ? = Undetermined
    * = Experiment ended at 12 mo
    0 = No cirrhosis

None of the control rats showed cirrhosis of the liver   In fact, among several thousand of our rats of all ages, both control animals and those fed substances

other than selenium, hepatic cirrhosis has been extremely rare, having been seen only two or three times and this in a slight or moderate degree.

DISCUSSION. The preceding tables and text show that rats fed selenium in a grain diet at concentrations as low as 3 and 5 parts per million develop severe lesions. Previous reports have not shown severe symptoms of chronic poisoning in rats when the ration contained 5 parts per million, or less, of selenium (5). On the addition of selenium to the diet there were two categories of rats, a more susceptible group which died within the first eight weeks, and a more resistant group which survived, in most instances, a year or more. In the more resistant group it is fair to assume that severe liver cirrhosis did not fully develop until later in the experimental period. If these rats had been killed within the first two months of the experiment, this degree of selenium poisoning might not have been observed. The proportion of resistant rats in each group depended on the concentration of selenium in the diet.

The data on growth rates show that the more susceptible group of rats on each concentration of selenium grew at a slower rate than the controls. The more resistant groups did not show a significant effect on growth during the first eight weeks of the experiment; however during the later period this group also grew more slowly than the controls. The restricted food consumption of the rats on the seleniferous diets may account for part of the retarded growth. However, since many rats at the lower concentrations of selenium showed severe lesions in the liver, this voluntary restriction of food consumption cannot be the only factor in the retarded growth rate.

The test with 3 parts per million of selenium from corn showed a greater toxicity than the similar test with 5 parts per million in the earlier experiment. The difference can be partly explained by the increased food intake of the rats on the lower concentration of selenium. It has been shown (11, 15, 16) that animal fed seleniferous diets voluntarily restrict their food consumption. In general, our food consumption records show that this restriction is in proportion to the amount of selenium in the diet. The rats on the 3 parts per million, therefore, received a relatively higher dosage of selenium than those on the 5 parts per million. Other factors, such as a decreased susceptibility of the rats in the later experiment and a possible change in the form of the selenium in the corn, may account for part of the increased toxicity. The selenium-bearing corn in both experiments came from the same batch which had been mixed thoroughly in a rotating mixer. Analyses showed that the selenium content of the corn had not changed during the two-year lapse in time between the two experiments.

SUMMARY

Rats fed selenium in a grain diet at concentrations of 3, 5, 7, 10, 20 and 40 parts per million showed toxic effects at all levels of selenium.

Selenium at the concentration of 10 parts per million, or more, with the exception of the 10 parts per million from the selenide, killed most of the animals within the first eight weeks. Lower concentrations of selenium produced chronic symptoms which included a decreased growth rate, a restriction of food consumption, and slight to severe pathological lesions.

Selenium at the concentration of 10 parts per million from the selenide was about half as toxic as the same concentration from wheat and corn

The outstanding pathological lesion was cirrhosis of the liver, seen in over 70 per cent of the rats surviving more than three months   Among 43 cirrhotic rats surviving 18 months or longer, hepatic cell tumors developed in 11 and marked adenomatoid hyperplasia in four others   Rats dying during the first three months showed a subacute type of liver damage   Lesions in viscera other than the liver were not characteristic or extensive

Female rats were more susceptible to selenium than male rats

Rats from different litters on the same dosage of selenium showed a significant difference in growth rates

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THE EVALUATION OF THE ANALGESIC ACTION OF PETHIDINE HYDROCHLORIDE (DEMEROL)

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Pethidine hydrochloride is the name which was suggested by the Pharmacopoeia Commission and approved by the General Medical Council (1) for 1-methyl-4-phenyl-piperidine-4-carboxylic acid ethyl ester hydrochloride. It was introduced into medicine in Germany under the trade name Dolantin; the trade name in Britain is Dolantal, in America Demerol.

The compound, primarily intended for use as a spasmolytic, was soon found to have considerable analgesic properties, and many authors have testified to its value in this respect (2). Although pharmacological studies of the compound have been made (3, 4, 5) we have seen no complete laboratory estimation of the relative value of the drug as an analgesic. Eisleb and Schaumann (3) reported that in white mice a fifth of the lethal dose caused complete lack of pain perception, but they gave no details of the method of testing which they used, nor do we accept their conclusion. Kiessig and Orzechowski (6) found that the drug had a marked analgesic effect in dogs suffering from experimental toothache. Sostmann (7) found it to be five to ten times as effective as aminopyrin in analgesia tests.

In a more recent paper Batterman and Himmelsbach (10) have compared pethidine with codeine and morphine on human subjects, using the elegant method of Wolff, Hardy and Goodell (11, 12). Their relative figures for the efficacy of the new drug are rather less favourable than those recorded here.

In this study a detailed comparison of Pethidine with morphine, diacetylmorphine, codeine and several of the compounds classed as "antalgiques" by Fourneau (8) has been made, using a new technique for the detection of analgesia.

EXPERIMENTAL. When this work was begun, the exact method of testing for the loss of pain perception had not been decided. As it was intended to use the same method for testing a number of synthetic compounds related to Pethidine, it was thought that the use of experimental animals was preferable to the use of human volunteers for the first tests. A number of methods which had been described previously were tried, but partly owing to inability to obtain consistent results with most of these, and partly because the only experimental animals obtainable in sufficient quantity at the time were mice, it became evident that a new technique was desirable.

After a number of methods of applying a painful stimulus to a mouse had been tried, stimulation by heat seemed to be most satisfactory, and an extremely simple apparatus was designed and made. This apparatus consists of a zinc plate which can be maintained at temperatures of 45°C. upward with an accuracy of ±0.5°C. On the zinc plate is a hollow glass cylinder, 15 cm. in diameter, into which the mouse is dropped, so that it is forced to stand or walk on the heated metal plate.

Various plate temperatures were tried, in observing the reactions of normal mice when dropped on to the plate. It was found that at temperatures up to 50°C. the mice reacted

irregularly some showing evident signs of discomfort within about twenty seconds while others seemed quite comfortable for much longer periods   At higher temperatures however, all mice reacted although there was a considerable degree of individual variation   It was found that at 55°C all tested animals reacted within thirty seconds and at 60°C all reacted within twenty seconds   The first signs of discomfort shown by a mouse on the hot plate are that it sits up on its hind legs and licks or blows its front paws to cool them   In a few seconds the pain is too great to be borne by the back paws and the mouse either kicks its legs and dances about the restraining cylinder or attempts to jump out of the cylinder   As normal mice often sit up and groom their front paws the movement of the hind limbs has been used as the criterion of acute discomfort

The standard time of exposure to the pain stimulus has been 30 seconds   The mice are tested at regular intervals after injection of the drug under test—at intervals of ten minutes for the first hour after injection then at intervals of twenty minutes for at least two hours or until the mouse is again sensitive to the particular pain stimulus used whichever period is greater   It has been usual to employ groups of ten mice at each temperature at each dose   The temperatures used have been 50°C to 70°C in steps of 5°C   Higher temperatures have not been used as there is the probability of damage to the feet with consequent altered threshold

The period of analgesia produced in each mouse can be estimated in this way with some degree of accuracy and the average duration of analgesia for each group of ten mice after injection calculated   Thus under A in tables 1 and 2 8/10 means that of the 10 mice tested with the drug at that particular dose level 8 showed analgesia in that they did not respond in 30 secs on the hot plate under B the figure gives the average duration in minutes of analgesia in the animals in which such response was established under C the figure is the standard deviation of the figures for which B is the average and under D the average delay before the onset of analgesia in minutes   There is considerable individual variation in response as will be seen from the large standard deviations from the mean durations of analgesia shown in table 1 and in table 2

The drugs used have been given in aqueous solution by subcutaneous injection under the skin of the abdomen   It is important that the same site of injection be used throughout, as preliminary experiments supported the view (9) that morphine and related drugs are more active in mice when injected under the skin of the back than when injected under the skin of the abdomen   Thus the L D 50 for Pethidine hydrochloride by subcutaneous injection in the mice we have used is 300 mgm /kgm under the skin of the abdomen 190 mgm /kgm under the skin of the back   Other workers have given lower figures for the L D 50 by subcutaneous injection in mice viz 150 mgm /kgm (3) and 160 mgm /kgm (4)   The mice used have been of uniform stock   Adults weighing 24 32 grams average 27 grams were used to avoid as far as possible differences in susceptibility due to age variations

Discussion   Each drug has its own characteristics, as follows

Morphine is an efficient analgesic against even severe pain stimuli if used in sufficiently large doses   It can produce long lasting analgesia, which reaches its maximum intensity about thirty minutes after injection   It is somewhat irregular in action, the individual variation in response being considerable

Diacetylmorphine is very much more regular in action with much smaller individual variation in response   The analgesia produced is at its maximum about ten minutes after injection

Codeine can produce long lasting analgesia if given in sufficiently large doses, which, however, approach the lethal dose   Variation in response is considerable   Maximum intensity of analgesia is reached about thirty minutes after injection

Pethidine can produce long lasting analgesia against mild pain stimuli, but

it is ineffective against severe pain stimuli of the type used.   Analgesia reaches its maximum intensity in about twenty-five minutes.

TABLE 1

DRUG	DOSE	55°C.				60°C.				65°C.				70°C.			
		A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
	mgm./kgm.																
Morphine hydrochloride	5	3/10	16	10	23												
	7.5	8/10	35	8	12.5	2/10	9	6	23	0/10							
	10	9/10	50	12	13.5	4/10	10	5	21	2/10							
	12.5	9/10	55	13	12.5	6/10	23	9	15	4/10	17	8	25	1/10			
	15	9/10	57	10	11	6/10	25	9	19	4/10	21	11	23	3/10	13	7	29
	20	9/10	90	15	7	8/10	37	10	16	7/10	32	9	20	3/10	17	10	27
	25	10/10	106	14	9	8/10	50	11	10	8/10	34	11	17	4/10	22	12	19
Diacetyl morphine hydrochloride	1	4/10	9	4	6												
	1.5	8/10	21	6	9												
	2	10/10	22	3	6	4/10	8	4	9.5								
	3	10/10	32	6	6	7/10	14	4	6								
	4	10/10	41	4	6	8/10	21	5	6	4/10	8	4	9.5	1/10			
	5	10/10	42	4	6	9/10	26	4	6	7/10	13	3	6	4/10	7	4	6
	7.5	10/10	51	4	6	10/10	33	3	6	7/10	22	7	6	7/10	16	5	6
	10	10/10	60	7	6	10/10	39	7	6	9/10	24	3	6	8/10	20	4	6
Codeine hydrochloride	30	5/10	13	5	12												
	40	6/10	17	6	14.5												
	50	6/10	25	9	9												
	60	7/10	29	8	14												
	80	8/10	44	11	14	4/10	8	4	18								
	100	10/10	61	8	10	5/10	23	13	22								
	125	10/10	79	12	10	5/10	48	19	11	2/10	10	8	18				
	150	10/10	89	14	7	7/10	57	17	8	4/9*	28	14	19	4/10	15	7	24
Pethidine hydrochloride	25	4/10	9	4	12												
	30	5/10	17	7	11												
	40	8/10	25	6	9	1/10											
	50	7/10	39	13	8.5	4/10	17	8	10								
	75	10/10	63	12	7	5/10	23	9	7	2/10							
	100	10/10	89	14	7	6/10	32	12	7	3/10	13	7	16				

"A" indicates proportion of animals in which analgesia was seen.

"B" indicates the average duration of analgesia in minutes.

"C" indicates the standard deviation of "B" $\left[\sqrt{\dfrac{\Sigma d^2}{n(n-1)}} \right]$.

"D" indicates the average time of onset of analgesia (minutes after injection).

* One animal of this group died within an hour of injection.

Phenobarbital sodium [*phenobarbitone* (B.P.)] had no analgesic action in doses lower than the anaesthetic dose. 50 mgm./kgm. produced hypnosis in two of the ten mice used, 75 mgm./kgm. in eight of the ten mice, 100 mgm./kgm. was

TABLE 2

| DRUG | DOSE | TEMPERATURE | | | | | | | | | | | |
| | | 55°C | | | | 60°C | | | | 65°C | | | |
		A	B	C	D	A	B	C	D	A	B	C	D
	mgm./kgm												
Phenobarbitone sodium (phenobarbital sodium)	50	0/10											
	75	0/10											
	100	1/10											
	200	1/10											
Aminopyrin	100	1/5											
	200	2/10											
	300	6/10	30	10	11								
	400	4/5	?										
Phenazone (antipyrin)	100	0/5											
	200	0/5											
	400	3/10	11	6	23								
	600	7/10	43	13	18 5	5/10	33	14	18	3/10	7	5	16
Aspirin	100	0/5											
	200	0/5											
	400	0/10											
	600	0/10											

"A" indicates proportion of animals in which analgesia was seen

"B" indicates the average duration of analgesia in minute

"C" indicates the standard deviation of "B" $\left[\sqrt{\dfrac{\Sigma d^2}{n(n-1)}} \right]$

"D" indicates the average time of onset of analgesia (minutes after injection)

Fig 1

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a strong hypnotic, and 200 mgm./kgm. was anaesthetic, taking full effect about half an hour after injection.
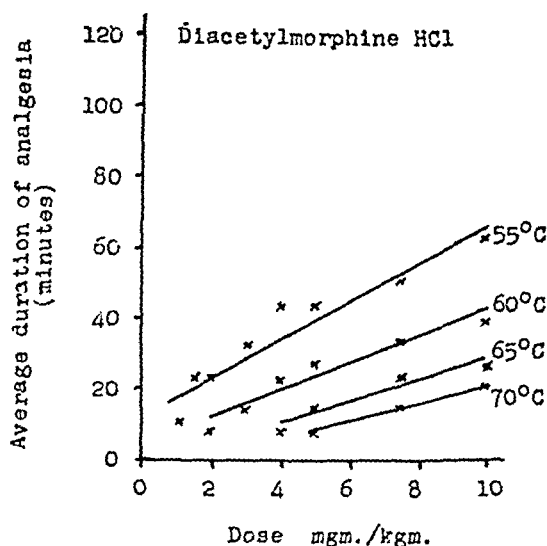
Fig. 2
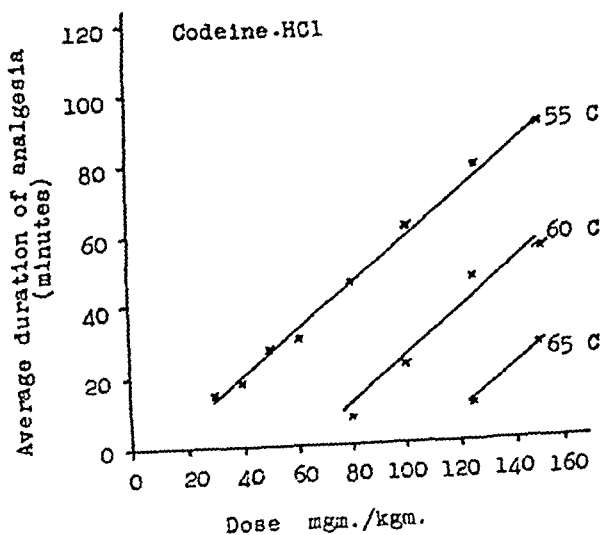
Fig. 3

Aminopyrin (Amidopyrin B.P.) had some slight analgesic action at 300 mgm./ kgm. With a dose of 400 mgm./kgm., however, the mice were too jumpy and

irritable for the test to be carried out   With 800 mgm /kgm three of the five mice injected died within twenty minutes, and one of the remaining two mice died within a day
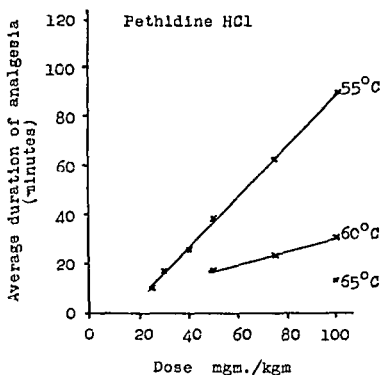
Fig 4

TABLE 3

Table of doses (mgm /kgm)

DURATION OF ANAL- GESIA	55°C				60°C				65°C				70°C			
	M	D	C	P	M	D	C	P	M	D	C	P	M	D	C	P
minutes																
30	7 5	3 2	57	44	17 0	6 4	109	97 5	21 2		155*					
60	14 2	9 3	100	72			155*									
90	20 7		148	102*												

*Extrapolated figures
M = Morphine HCl
D = Diacetylmorphine HCl
C = Codeine HCl
P = Pethidine HCl

Antipyrin (Phenazone, B P) had some analgesic activity in very large doses, the effect reaching its maximum in about twenty minutes   It is less potent than aminopyrin

Aspirin (Dissolved with the aid of twice its weight of sodium citrate) had no analgesic action in the doses used   With 600 mgm /kgm four of the ten mice were in convulsions ten to fifteen minutes after injection   With 800 mgm/kgm

two out of five mice were in convulsions within ten minutes of injection and one more within forty minutes of injection. Two of the mice died about an hour and a half after injection.

In figures 1 to 4 the average duration of analgesia for each group of mice is plotted against the dose of pethidine, morphine, diacetylmorphine and codeine hydrochloride. It will be seen that there are straight line relationships between dose and duration of effect within the limits of these experiments.

In table 3 the doses of each drug necessary to produce analgesia lasting thirty, sixty, or ninety minutes at each temperature have been read from figures 1 to 4, and in table 4 these figures have been reduced for comparison, morphine hydrochloride as the standard analgesic drug being used as the unit. It will be seen that morphine hydrochloride is about seven times as active as is codeine hydrochloride, and about one half as active as is diacetylmorphine hydrochloride. Pethidine hydrochloride has between one fifth and one sixth of the action of morphine hydrochloride against mild pain stimuli, but as has been mentioned,

TABLE 4

Ratio of doses in table 3

(Morphine HCl = 1)

DURATION OF ANALGESIA	55°C.				60°C.			
	M	D	C	P	M	D	C	P
minutes								
30	1	0.43	7.6	5.9	1	0.38	6.4	5.7
60	1	0.65	7.0	5.1				
90	1		7.1	4.9				

M = morphine HCl.
D = diacetyl-morphine HCl.
C = codeine HCl.
P = pethidine HCl.

it is quite ineffective against more severe pain. As it is a spasmolytic too, it may be more valuable against pain produced by, or due to, spasm.

The comparative figures for morphine, diacetylmorphine, and codeine agree very well with results obtained by other methods and with clinical experience, and this supports our belief that the method used is reliable as well as simple.

SUMMARY

1. A new method for the evaluation of analgesics is described.

2. By the use of this method, comparison of morphine, diacetylmorphine, codeine and pethidine hydrochlorides has been made.

3. Pethidine hydrochloride has been found to possess one fifth to one sixth of the analgesic activity of morphine hydrochloride against mild pain stimuli, but to be ineffective against severe pain.

4. Aminopyrine and antipyrin are effective only in very large doses. Phenobarbitone sodium and aspirin have no analgesic action that can be detected by this method.

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PHARMACOLOGICAL ACTION OF THE VENOM OF LATRODECTUS MACTANS AND OTHER LATRODECTUS SPIDERS

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Poisoning caused by the bite of spiders of the genus *Latrodectus* has been known since olden times (6) It is of frequent occurrence in the United States, Chile, Columbia, Guatemala, Mexico, Uruguay and Argentine (*Latrodectus mactans*), in Australia and New Zealand (*Latrodectus hasseltii*), in Italy, Spain and Russia (*Latrodectus tredecimguttatus*), in South Africa (*Latrodectus indistinctus*), and in Madagascar (*Latrodectus menavodi*) In Argentine many hundreds of cases have been reported (*Latrodectus mactans*) and we have recorded some 300 in the provinces of Buenos Aires and Santiago del Estero alone Generally it happens to rural laborers, who, with exposed chests, handle hay or load cereal bags At present, it is seen not infrequently among urban residents (9)

Eight species of this genus have been described in Taxonomy 1) *Latrodectus pallidus* (Cambridge, 1872), 2) *Latrodectus hystrix* (Simon, 1889), 3) *Latrodectus tredecimguttatus* (Rossi, 1770), 4) *Latrodectus geometricus* (Koch, 1841), 5) *Latrodectus foliatus* (Mello Leitao, 1940), 6) *Latrodectus concinnus* (Cambridge, 1905), 7) *Latrodectus indistinctus* (Cambridge, 1905), and 8) *Latrodectus mactans* (Fabricius, 1775) The first, second, fourth and eighth species are considered the fundamental ones (2) In our investigations we have used some 12,000 spiders of the species *Latrodectus mactans* (Gerschman and Schiapelli) Some experiments have been made using *Latrodectus geometricus*

The bite of *Latrodectus mactans* causes in man a typical syndrome from which the diagnosis can readily be made, even without knowledge of the bite With the bite a small puncture can be seen After a latent period of about a quarter of an hour, a burning pain is felt in the bitten area and this soon spreads over the whole body The pain becomes of an agonizing character and is accompanied by a state of mental irritability and restlessness In addition, tremors and clonic contractions are noted, appearing first in the bitten area and later extending throughout the whole body Pain and neuromuscular manifestations are intermittent and slowly grow in intensity As the poisoning progresses, there come spasmodic movements of the legs, abdominal rigidity, marked salivation and lacrimal secretion, exhausting sweating, the sensation of precordial oppression and fear of imminent death There is noted hyperesthesia, exaggerated reflexes, usually tachycardia and hypertension Among other effects very frequently observed are intestinal and vesical palsies, albuminuria and oliguresis Occasionally priapism, seminal emissions and enuresis occur All of these symptoms lessen in a few hours but return to some degree on the following days, lasting altogether for from 48 to 72 hours Throughout the poisoning, the local effects are insignificant—a small red puncture spot and sometimes a localized edema

The usual treatment has been purely symptomatic. The patient is kept at rest in bed, and morphine given for the relief of pain, reinforced by external heat or hot baths. Large amounts of fluid are given, and cardiac stimulants, diuretics and cathartics administered when indicated. The intravenous injection of calcium gluconate (10 cc. of a 10% solution) is reported useful (Hodges 3, Young 13).

On account of the inadequacies of symptomatic treatment, studies have been carried out with the purpose of producing an antitoxin serum. A horse was immunized with subcutaneous injections of *Latrodectus mactans'* cephalothorax triturated and suspended in saline. (Pirosky, I., Sampayo, R., and Franceschi, C. 7 and 8). The injections were made with a relatively small dose for a period of 14 weeks and with a high dosage for the following 7 weeks. The serum obtained from the horse was treated in two ways—by precipitation with sodium sulphate giving a purification index of 1.35, and by digestion with pepsin (Pope's method) with a purification index of 6. One cc. of the latter protected 50% of the white mice against 3 minimal lethal doses. One gram of proteins obtained by precipitation protected against 49.5 M.M.D. This serum has a high preventive and curative power for the poisoned Guinea-pigs, but offers no protection against the venom of *Ctenus nigriventer* and *Lycosa raptoria*. Here there is no complement fixation and no precipitins are found. We have also prepared a standard "test serum" according to the technique of the Instituto Bacteriologico del Departamento Nacional de Higiene.

We have used the serum in treatment of patients in dosage of 10 cc. given subcutaneously (7). In the few cases in which it was given, all the toxic manifestations had disappeared within three hours after the injection. In very serious cases, the intravenous route might be used, after having tested the subject's sensitivity intradermally.

Pharmacological Actions. The pharmacological actions of spider venom have been studied by a number of investigators: Houssay and Negrete (4), Troise (11, 12), D'Amour et al. (1) with *Latrodectus mactans*; Kellaway (5) with *Latrodectus hasseltii* and Shapiro et al. (10) with *Latrodectus indistinctus.*

The work reported here deals especially with the action of the venom of *Latrodectus mactans* on the central and peripheral nervous system and on striated and non-striated muscle of various animal species. The venom was obtained from the glands without chelicerae. Varying quantities of the glands were *triturated* in saline solution and the whole opalescent suspension injected without filtering.

The striking effects of the venom are due mainly to actions on the central nervous system. These and other actions studied are *described under separate headings.*

Actions on the Motor System. We were able to find changes in the motor system in all the animals experimented upon: a) initial trembling in Guinea-pigs, and also in dogs after an intravenous injection of venom; b) contractions of the skeletal muscles and stiffness of the abdominal wall (Guinea-pigs, cats and dogs); and c) non appearance of these phenomena in denervated muscles.

Troise (11) proved that the intraspinal injection of venom in decapitated frogs

produced generalized trembling and muscular rigidity. We have been able to prove a similar but more intense symptomatology in spinal dogs.

The central nervous origin of the nervous manifestations caused by injection of the venom has been shown by the registration of the electrical phenomena produced by the spontaneous activity of the cerebral cortex, spinal cord, nerve and muscles.

The electroencephalograms of Guinea-pigs—nembutal anesthesia—showed after the injection of the venom, *acceleration of the fundamental rhythm* (from 4–5 p.s. to 20–25); *a rise of its amplitude* (from 45 to 60 microvolts) and the *appearance of rapid spicae*. The amplitude of the oscillations diminishes secondarily. The effects come on in a couple of minutes after intravenous injection but only after about fifteen minutes following subcutaneous injections. The injection of the extract from one half or one gland will produce the effects. The intravenous injection of large doses, an extract of 10 glands, causes bronchial obstruction and asphyxia which in themselves produce alterations in the electrical activity.

These electric manifestations gradually disappear following the intravenous injection of anti *Latrodectus* serum, as shown in figure 1.

Intravenous injection in a nembutal-pentothal anesthetized cat produced intensification of the electrical activity of the brain, shown by the appearance of waves of greater amplitude and frequency, 18 minutes after the injection. These began to diminish 25 minutes later and become similar to those present before the intoxication 45 minutes afterwards.

Similar phenomena have been observed on registering the electric activity of spinal cord, nerve and muscle; speeding up of spinal rhythm, appearance of waves in nerve and spicae in innervated muscle. These changes are visible 5–10 minutes after the injection and increase up to 20 minutes decreasing after this period (fig. 2).

In decerebrated or in spinal animals, the spinal nervous and muscular activity is more intense than in intact animals, especially the spinal activity.

If electrodes are placed on the head and both hind-legs, one of which is totally denervated, the appearance of the electroencephalographic modifications in the cortex is followed by the production of small spicae in the innervated muscle, while no modification is observed in the denervated leg (fig. 3). In 18 minutes after the intravenous injection of extract of 5 glands, real volleys of spicae are observed in the innervated leg. Decapitated dogs or cats show similar phenomena.

Action on striated muscle: D'Amour et al. investigated the action of the venom of *Latrodectus mactans* on neuro-muscular preparations of toads. The authors conclude that the effect of the venom on muscle is not direct but comes from the action on the central nervous system. We have confirmed D'Amour's results. Preparations of Sartorious muscles of toads (*Bufo arenarum*) placed in Ringer's solution containing the venom (1 mg. of the gland to 10 cc. Ringer's) showed no contraction.

Sensory System. Hyperesthesia and paresthesia are common in human beings bitten by the spider. The most striking manifestations of the syndrome caused

by the bite, however, are the wide spread pains, along the spine, in the muscles, in the joints. In our experimental animals following venom injection, hyper-esthesia was present and we have commonly observed manifestations of in-tensive pain——cries or wails.
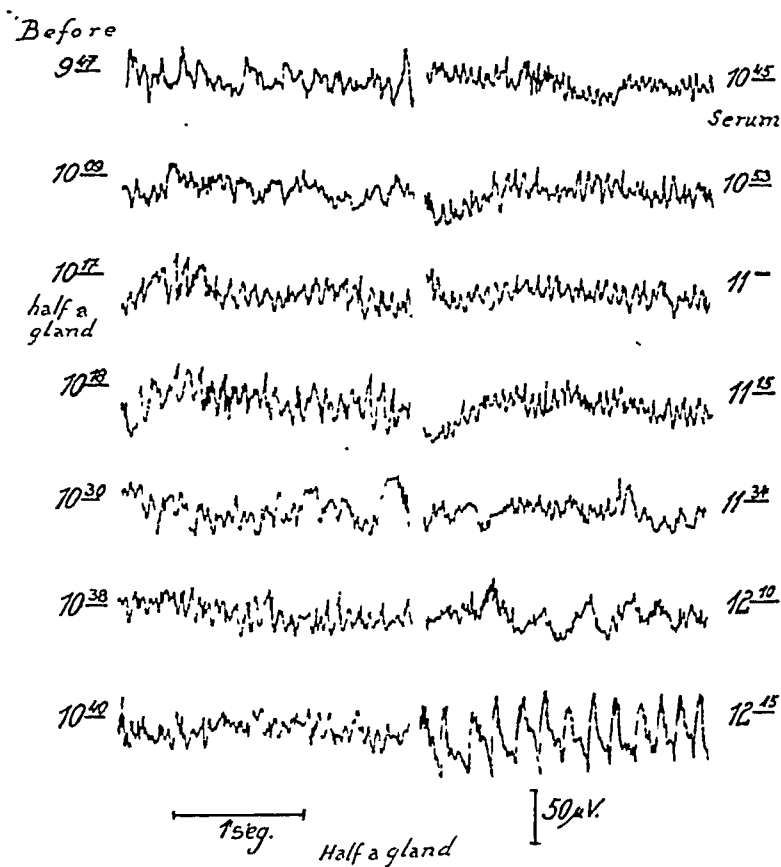
FIG. 1. ELECTROENCEPHALOGRAM OF GUINEA-PIGS (J. AND K.) ABOUT 300 GRAMS
At 10 h. 17 min. intravenous injection of half a gland   28 minutes later, injection of 1 cc. of serum.

Autonomic Nervous System. The exciting action of the venom on the central nervous system is transmitted to the peripheral effectors not only by the motor nerves but also through the automatic ganglionar system. The effects produced through the sympathetic system are specially striking

Arterial pressure: The intravenous injection of venom produces a rise of ar-terial pressure. This hypertensive action is not modified by atropine, but is
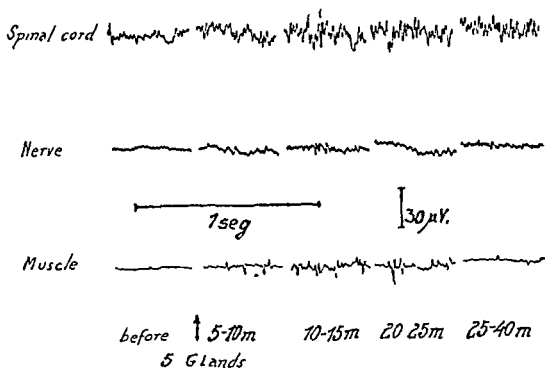
FIG 2 CAT 2 5 KG , ELECTRIC PHENOMENA IN SPINAL CORD NERVE AND MUSCLE, BEFORE AND AFTER INTRAVENOUS INJECTION OF 5 GLANDS
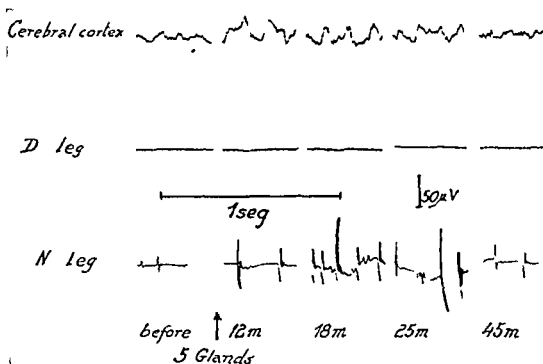
FIG 3 CAT 3 KG INJECTION OF 5 GLANDS ELECTRIC PHENOMENA OF CORTEX MUSCLES OF DENERVATED LEG (D LEG) AND MUSCLES OF NORMAL LEG (N LEG)

Note that denervated muscles do not show electric modifications, while these are evident in the simultaneous electroencephalogram

strongly reinforced by cocaine and is diminished or abolished by Fourneau 933 (sympatheticolitic agent) The hypertension is due to a generalized vasoconstrictory action and is observed in the absence of the adrenals, the kidneys or

all abdominal viscera. There is a central nervous factor and a humoral accessory one (see action on circulation).

Secretion of adrenalin: The venom has no significant effect on adrenalin secretion as shown through adrenojugular anastomosis and by effects on the nictitating membrane.

Action on the nictitating membrane: In adult cats of 2.2 to 5 kg. weight, under nembutal and sodium pentothal anesthesia, the eyeball was extirpated and the nictitating membrane attached to a writing lever. The intravenous injection of the extract of 3 glands produced a slow and delayed contraction of the membrane, coming on 6 minutes after the peak rise of the arterial presure. This action did not occur with the same or a larger dose (extract of 5 glands) following
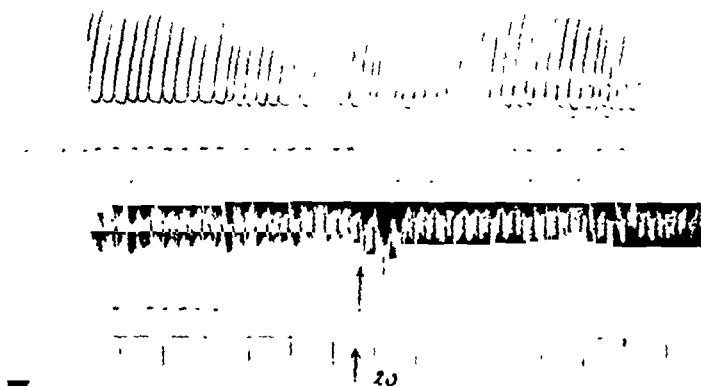
FIG. 4. PREVENTIVE ACTION OF ANTI-LATRODECTUS SERUM. DOG 7, 4 KG., AMYTAL
From top to bottom: *Neumogram, carotid pressure, time in minutes.* No modifications on tracings noticeable, save a slight fall of pressure, possibly due to reflexes caused by injection. Initial pressure: 90 mm. Hg.

extirpation of the superior cervical sympathetic ganglion, although here 1 microgram of adrenalin injected intravenously produced an immediate contraction.

These results show that if there was any increase in adrenalin secretion, this was too small to be effective.

Pupil: Pupillary dilation was seen in toads following intravenous injection of the venom. It is also seen in cats, but not after extirpation of the superior cervical sympathetic ganglion.

Color of skin: Pallor of the skin of toads (*Bufo arenarum* Hensel) was produced by the intravenous injection of venom. The pallor corresponds to that produced by central nervous stimulation, restraint, operations, light and injection of adrenalin.

Sympathin: The intravenous injection of venom produces a decrease in the volume of the spleen, even when this is denervated and when the two adrenals have been excised. This suggests a possible liberation of sympathin or a direct

action of the venom, or both   Contraction of the denervated spleen is slowed by Fourneau 933, but is not suppressed (fig 6)

Latent period The latent period which always exists between the intravenous injection of the venom and the occurrence of its effects, is very striking   This suggests the possibility of a slow central nervous penetration or of a chemical transformation

FIG 5 POTENTIATION THROUGH COCAINE (1 CG PER KG) OF HYPERTENSIVE ACTION OF
LATRODECTUS MACTANS VENOM

Dogs 6-7 kg anaesthetised with nembutal time in minutes   Injection of 15 glands each
In both cases cocaine potentiated the hypertensive action of the venom   Compare with figures 6 and 7

Action of adrenalin In one experiment, after injecting venom we observed an inversion of the bronchial action of adrenalin   (Bronchoconstriction) Other experiments demonstrated that adrenalin increased arterial pressure after the intoxication but we did not notice any qualitative or quantitative modification of its action as reported by Shapiro et al (10)

ACTION ON THE CIRCULATORY SYSTEM   *Action on arterial pressure* Shapiro et al (10) experimenting with the venom of *Latrodectus indistinctus* found in rabbits and in anesthetized and in decapitated cats a rise of blood pressure

Troise (11) observed that venom of *Latrodectus mactans* injected intravenously in anesthetized dogs produced strong and prolonged elevation of the arterial
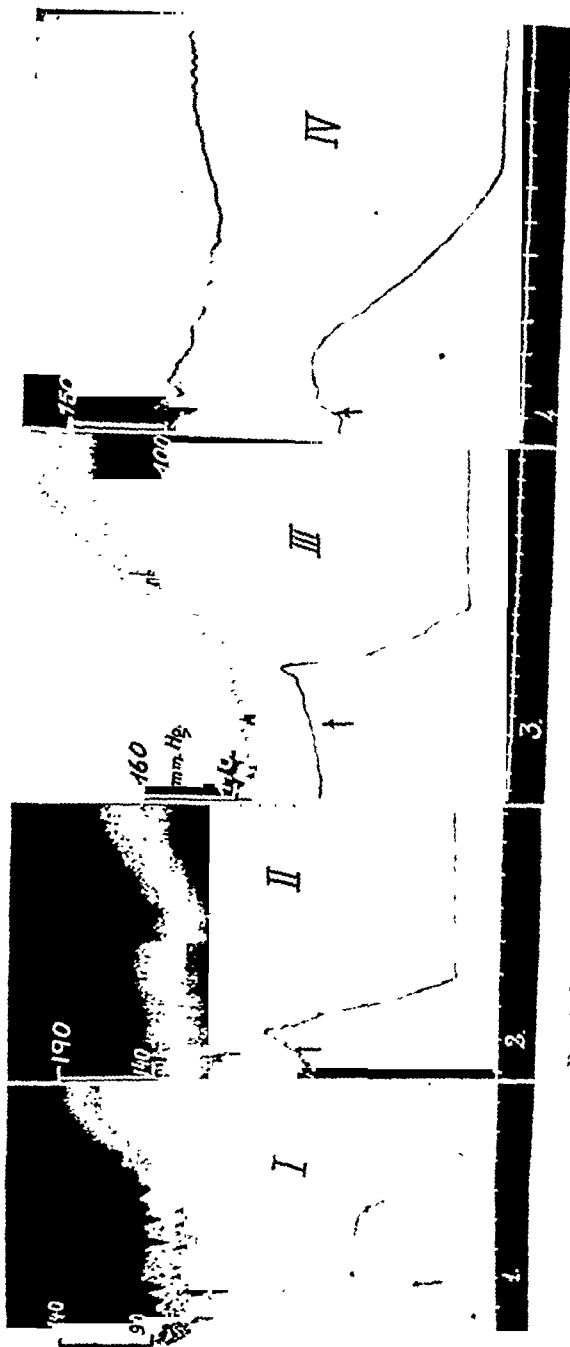
Fig. 6. Dogs of 11, 7, 8 and 8 Kg. respectively, Nembutalized

Action over plethysmograms of spleen: In the four graphics from top to bottom: Carotid pressure, plethysmogram of spleen, time in minutes.
Graphic I: Normal spleen, in 1, 15 glands in 10 cc.
Graphic II: Denervated spleen sensitive to 0,000005 grams adrenalin; in 2, 15 glands.
Graphic III: Denervated spleen in dog without adrenals. Sensitive to 0,000001 gram adrenalin. In 3, 15 glands.
Graphic IV: Denervated spleen, previous injection of F.933 (10 mg. per kg) which inverted the action of adrenalin. In 4, 10 glands.

pressure He considered that the hypertension was probably of central origin
However, a new sample of venom, in spite of being more toxic, had little hypertensive power From this he inferred that the hypotensive effect was due not
to the neurotoxin in the venom but to another constituent, quite separate from
the toxic factor

We have carried out experiments with cats, dogs and rats and have observed
in all of them hypertensive action with different samples of glands of *Latrodectus*

TABLE 1

Hypertension in dogs

EXPERIMENT		WEIGHT	GLANDS	PRESSURES	LATENT PERIOD	MAXIMUM OF THE CURVE
		kg		mm Hg	m nutes	m nutes
1/VII/42		9 8	20	100–160 60		10
4/VII/42		8 2	30	100–156 56		4
8/VII/42	Serum previous to injection of venom	7 1	20	90– 90 0		
8/VII/42		19	15	150–214 64		6
id id		11 5	15	91 153 62	3	11
id id		9	20	90–150 60	½	5
10/VII/42		9	20	95–150 60	½	5
10/VII/42	Decapitated	10 5	15	70– 80 10	5	2
10/VII/42	Without adrenals	8	15	85–119 34	½	8
13/VII/42		11	15	90–140 50	4	7
14/VII/42		7	15	140–176 36		?
14/VII/42	With Γ 933	7 5	15	100–124 24	4	7
15/VII/42		8	15	110–222 112	3	12
15/VII/42		20	15	200–248 48	3	9
16/VII/42		11	15	90–122 32	3	7
21/VII/42		12	15	90–122 32	2	9
28/VII/42	With Γ 933	8	10	?	?	?
1/VIII/42	Femoral	12	15	80–116 36		6
7/VIII/42	With F 933	5 2	15	100–122 22	4	8
8/VIII/42		9	15	98 120 22	2	?
18/VIII/42	Eviscerated	10	15	150–216 66	1½	4
18/VIII/42	Without vagus and carotid plexus	8	15	55–145 90	1½	7
22/VIII/42		6 5	20	75–163 88		8
7/X/42	Cocaine	6	15	130–230 100	1	10
7/X/42	Cocaine	7	15	125–215 90	1	10
7/X/42	Atropine	5 7	15	80 120 40	2	9

mactans kept in sulphuric vacuum at low temperature The extent of the rise
in pressure was not uniform with different samples of venom, which gives support
to Troise's conclusion that it is due to other than the neurotoxic principle

I Hypertension in dogs Of 25 animals weighing between 5 and 20 kg all
of them injected intravenously with a macerate of glands in a proportion of
15 to 30 glands for 10 cc of saline, only a single animal did not respond with
rise of arterial pressure (See table 1) Rises ranged from 10 to 112 Hg mm ,

beginning in some cases nearly immediately after the injection, but in most, after a latent period, which varied from 30 seconds to 4 minutes. The curve reached its highest point between 6 and 9 minutes, beginning to drop afterwards and reaching its original level between 15 and 20 minutes after the injection.

II. Hypertension in cats: In two experiments we observed a similar curve to that found in dogs. Shapiro et al. (10) described an initial drop of pressure which we did not notice.

III. Hypertension in rats: In two experiments—with white rats of 200 grams of weight, anesthetized with nembutal—blood pressure rose immediately after intravenous injection of extract of 4 glands in 0.75 cc. of saline. This rise was followed by a drop and then by another slower rise reaching its peak 5 minutes after the injection. The increases were as high as 40 mm. Hg.
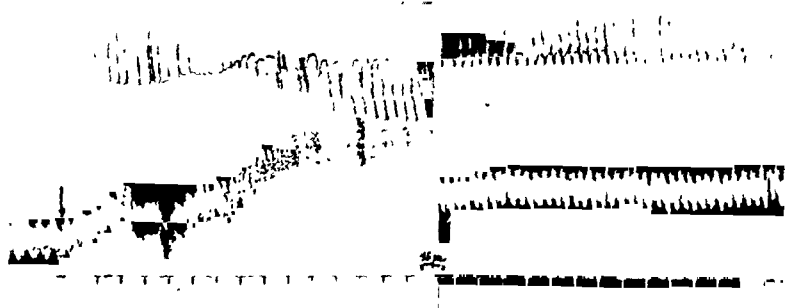
FIG. 7. DOG 9, 8 KG., AMYTAL
From top to bottom: *Neumogram, carotid pressure, time in minutes.* As a consequence of the first injection of 20 glands a rise of arterial pressure and modifications of respiratory rhythm were observed. After normalization of the dog, the reinjection of the same dose produces none of the former actions. Initial pressure: 105 mm. Hg.

Tachyphylaxis: We have often observed that the repetition of the toxic doses or the injection of still larger doses had no pharmacological effect whatsoever. (See fig. 7.)

Action of the anti-LATRODECTUS serum: After the intravenous injection of 1 cc. of anti-*Latrodectus* purified serum the administration of venom from 20 glands produced neither hypertension nor any change in respiratory rhythm in an 8 kg. dog. No alterations in the electrocardiogram were noted (see fig. 4).

On injecting 1 cc. of serum at the peak of the hypertensive curve, when alterations of the neumogram were already noticeable, a drop in arterial pressure and return to normal of respiratory movements followed.

Mechanism of hypertension. a) *Central nervous system:* The decapitated animal showed the typical rise of blood pressure on the injection of *Latrodectus maclans'* venom.

It was observed also after extirpation of the carotid sinus and section of both vagi. The direct action on the spinal cord is shown by these experiments.

b) *Role of the splanchnic:* Eviscerated animals (without gut, kidneys and

liver) also showed increase of pressure after the injection of venom This suggests
that the hypertension is not due only to the contraction of the splanchnic area

c) *Adrenals and adrenalin secretion* The rise of arterial pressure is not due
to a discharge of adrenalin, as it is seen in animals after extirpation of the adrenals
(3 experiments) Further, with suprarenojugular anastomosis (dogs) we did
not find adrenalin discharge

d) *Reflex and peripheral excitability of vasomotor nerves* Troise (12) studied
whether the venom modified the reflex excitability of vasomotor nerves and the
action of direct excitability of the great splanchnic over the arterial pressure
While the action of the venom lasted, he observed a marked increase of the
reflex hypertensive action produced by the excitation of the central vagus,
otherwise hypertensions produced by central excitation of the sciatic nerve and
peripheral excitation of the great splanchnic and vagus did not suffer noticeable
modifications

e) *Production of renin or hypertensine* Because the curve of hypertension
resembles the one produced by the injection of renin, we investigated whether
the increased pressure could not be produced by production of hypertensine
Incubation "in vitro" of venom with hypertensinogen did not produce hyper-
tensine The hypertension is not due to liberation of renin since it was evident
in animals deprived of kidneys

f) *Atropine, Fourneau 933, Pitressin, Cocaine, Adrenalin* The hypertensive
action is not prevented by the injection of Atropine (1 mg × kg), Fourneau
933 did not prevent but diminished the increase of arterial pressure, in our 3
experiments During the venom action Pitressin produced its customary effect
On the other hand the previous injection of Cocaine (1 cg × kg weight) rein-
forced the hypertensive action of the venom (see fig 5, two experiments)

g) *Latent period* The existence of a latent period between the injection and
the appearance of the different pharmacological actions of the venom, makes
plausible the hypothesis of the freeing of a substance, through catalytic action
of the venom

h) *Plethysmograms of the spleen* In Troise's (12) experiment the plethysmo-
gram of the spleen showed no modification, but he used venom without hyper
tensive action We have made plethysmographic records of the spleen in
dogs, anesthetized with nembutal, registering at the same time the arterial
pressure

The spleen contracted intensely shortly before the end of the latent period,
preceding the rise of the arterial pressure (see fig 6) This contraction was also
observed in dogs with denervated spleens (fig 6, II), proving a direct humoral
action on this organ This can not be explained by adrenalin secreted by the
adrenals, since it was observed in animals with bilateral adrenalectomy (fig 6,
III) It is difficult to prove what humoral factor is involved in the contraction
The effect might be due to sympathin formation for following the injection of
Fourneau 933 (10 mg per Kg) the spleen contracted (fig 6, IV) The contrac
tion however came on more slowly than before It is possible that the venom
has a direct action on the spleen and that the contraction represents a combined
sympathin and direct action

i) *Plethysmograms of the gut:* In our experiments we have found passive dilatation of the gut, which might be referred to direct action of the venom or, with more probability be a consequence of the rise of portal pressure, which we did not register.

j) *Plethysmograms of the kidney:* In one experiment, we have obtained passive renal dilatation coincident with the rise of arterial pressure. In another the kidney contracted after a latent period of two minutes, preceding shortly the beginning of hypertension. In both cases there was suppression of diuresis produced previously by the injection of hypertonic glucose solution.

Action on the heart. Isolated heart: The effect of the venom of *Latrodectus indistinctus* on the hearts of cold blooded animals (Xenopus laevis and Bufo arenarum) and in mammals (cats and rabbits) has been studied by Shapiro, Sapeika and Finlayson (10). The movements in the cat's heart were registered by the Cushny myocardiograph and the electrocardiograph. Among the effects noted were decrease in amplitude of contractions, irregularities, auricular fibrilation, and heart block. An inversion of the T wave was shown in the electrocardiogram.

Using the electrograph, we have investigated the action of the venom of *Latrodectus mactan's* and of the anti-*Latrodectus* serum on dogs and Guineapigs. In dogs there was found a reduction of voltage of R, an elevation of the ST segment and laterations of T, which changed from bifasic to positive in Lead II and Lead III. These alterations did not occur if an injection of 1 cc. of anti-*Latrodectus* serum was given previously. With Guinea-pigs we observed, in every case, a change in direction of the T wave, which as in the case of the dog was prevented by a previous injection of serum. In one experiment, injection of venom from 0.5 gland intravenously produced a change of ST and of T which from 0.5 mm. positive and 1 mm. negative respectively were transformed into isoelectric. The injection of 0.5 cc. of serum caused the T wave to turn negative after 2 minutes; with rapid improvement of the animal. 30 minutes after the injection of the serum we obtained again $ST = +0.5$ and $T = -1$ mm.

Action on Respiration. In the Guinea-pig the venom induced bronchial contraction. In the dog most of the respiratory changes recorded were due to muscular contractions of the thorax and abdomen.

In a dog receiving the venom of 30 glands, irregular breathing, with strong increase in respiration movement, alternating with periods of apnea were observed 4 minutes after the injection, concomitant with the peak of blood pressure rise. Generalized tremors and muscular contractions were also present. After injection of 1 cc. of anti-*Latrodectus* serum all these actions of the venom disappeared. Another dog was given 1 cc. of the serum and 22 minutes later the venom of 20 glands. There was no modification of the pneumogram in this case (fig. 4).

Action on Involuntary Muscular Organs. We were not able to find any action of the venom on the following involuntary muscular organs: Bronchia of Guineapig, bronchia of dog, isolated gut of Guinea-pig and non-pregnant uterus of rabbits.

Action on Blood Composition. In two dogs, one unanesthetized and one anesthetized with nembutal, the venom of 20 glands caused an increase in blood sugar from 67 to 112 mg. per cent and from 94 to 120 mg. per cent respectively. The venom was not haemolytic. Changes in blood P, K, Ca, Na and Cl were too small to have significance.

SUMMARY AND CONCLUSIONS

1. The occurrence and symptoms of poisoning from the bite of *Latrodectus mactans* are described.

2. The toxic substance is present in the glands of the spider and can be extracted by trituration of the gland in saline solution. An antitoxic serum has been obtained by immunizing a horse with the venom.

3. The venom contains a neurotoxic substance which has a diffuse excitatory action throughout the entire central nervous system, shown by electrical registration of the cortex, spinal cord and motor nerves. The isolated Sartorious muscle of the toad and denervated mammalian muscle are not affected by the venom.

4. The excitation of the central nervous system involves the autonomic system. There is a rise in arterial pressure due to generalized vasoconstriction. It is not affected by atropine, but strongly reinforced by cocaine and diminished or abolished by Fourneau 933. The rise is present after removal of the adrenals, the kidneys or all abdominal viscera and after decapitation and extirpation of the carotid sinus and section of both vagi. Contraction of the nictitating membrane and dilatation of the pupil are caused by the venom but are absent after extirpation of the superior cervical ganglion.

5. The venom does not produce a significant increase in adrenalin secretion. It causes a decrease in the volume of the spleen, even when this is denervated, suggesting a possible liberation of sympathin or a direct action on this organ.

6. Abnormalities in respiration are produced by the venom but these are results of thoracic and abdominal muscle spasms.

7. The venom has no effect on the involuntary muscles tested, bronchioles, intestine and uterus.

8. Tachyphylaxis is seen with the venom in that repetition of dosage is without pharmacological effect.

9. The intravenous injection of the antitoxic serum prevents or abolishes the poisonous effects of the venom.

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THE EFFECT OF TEMPERATURE ON THE INACTIVATION OF EPINEPHRINE IN VIVO AND IN VITRO[1]

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It was shown by Trendelenburg (1) that with small doses of epinephrine there was a close parallelism between the increase in blood pressure and the concentration of the hormone in the arterial blood   The effects of such doses were quite transitory and when the arterial pressure returned to the control level the injected epinephrine had disappeared from the circulating blood   The rapid disappearance of epinephrine from the blood of the intact animal can best be explained by assuming that the epinephrine molecule is changed to a pharmacologically inactive form in the tissues   However, it is likely that this inactivation is a complex process because it can be retarded by a variety of substances (amino acids, ascorbic acid, glutathione) which probably affect different reactants or reactive groups (for reviews cf Trendelenburg, (2), Bernheim, (3), DeMeio, (4)   On the basis of recent evidence there are at least three possible enzymic mechanisms for the inactivation of epinephrine *in vivo*

(a) Oxidation of the hydroxy groups by the cytochrome system (5) or by polyphenol oxidase (tyrosinase) (6) to give the pharmacologically inactive pigment adrenochrome   This unstable substance is slowly oxidized to a melanin (5)

(b) Oxidative deamination of the sidechain, catalyzed by amine oxidase (7, 8) to yield the corresponding aldehyde and methylamine (9) which are also pharmacologically inactive

(c) Esterification, followed by excretion of epinephrine as the inactive sulphate ester Esterification appears to occur chiefly in the liver where it is catalyzed by a "sulfosynthase' (10, 11, 12)

The relative importance of these mechanisms in the inactivation of epinephrine *in vivo* is not yet clear (cf Richter and MacIntosh, 11, Alles, Blohm and Saunders, 13, Philpot and Cantoni, 14, Richter and Tingey, 15, Torda, 16, and the reviews of Bernheim, 3, and DeMeio, 4)

In the course of studies on hypothermia in mammals the question of the effect of lowered body temperature on the inactivation of epinephrine arose   It has been reported that the action of the hormone is prolonged at reduced body temperatures (17, 18, 19) but the data available are not very convincing   The results of two lines of investigation are reported in the present paper, (1)   A study of the effect of temperature on the rate of oxidative deamination of epinephrine by amino oxidase *in vitro*, (2)   A study of the response of the acutely denervated nictitating membrane of the cat to the administration of epinephrine

at rectal temperatures ranging from 12.9° to 42.3°C. This part includes an investigation of the effect on epinephrine inactivation of warming the region of the liver during general hypothermia.

PART I. THE EFFECT OF TEMPERATURE ON THE OXIDATIVE DEAMINATION OF EPINEPHRINE BY AMINE OXIDASE IN VITRO

Although it is not yet clear whether the inactivation of epinephrine *in vivo* is brought about by amine oxidase or by other enzymic mechanisms, it has been shown that epinephrine is rapidly inactivated *in vitro* by amine oxidase (7, 8). The effect of temperature on the activity of this enzyme with epinephrine as substrate was determined as a background for the experiments on intact animals reported in Part II.

METHODS. A preparation containing amine oxidase was made from rat liver by the method of Richter (9). Oxygen consumption was measured manometrically in a constant volume type Warburg apparatus. The main chamber of the reaction vessels contained 1.0 ml. of enzyme preparation, 0.1 ml. of 2 M. semicarbazide and 0.5 ml. 0.1 M phosphate buffer, pH 7.3. After thermoequilibration, 0.2 ml. M. sodium cyanide and 0.2 ml. of 1:1000 1-epinephrine[2] in 0.1 N hydrochloric acid were added at the same time from the two side arms. The pH after such addition was still 7.3. Rates of oxygen consumption (in μl., N.P.T., per ml. enzyme solution per hour) were calculated from integral curves constructed on the basis of 5 or 10 minute readings. During the first 20 to 40 minutes of a run the total oxygen consumed was a linear function of time, and all oxygen consumption figures were derived from measurements during this period. Four constant temperature baths were available. This made it possible to carry out measurements at ten different levels of temperature with a given enzyme preparation during a period of eight hours after preparation. After this, the enzyme preparation was discarded.

RESULTS. The results of a representative series of experiments with one enzyme preparation are presented in figures 1 and 2. In figure 1, oxygen consumption is plotted as a function of temperature over the range 5° to 45°C. It is evident that under these conditions the maximum activity of the enzyme occurs at or above 45°C. During the period of observation there appeared to be no heat inactivation of the enzyme at or below 45°, the highest temperature used. The relation shown in figure 1 is of the continuous, regular type which is characteristic of metabolic reactions (20).

Various methods have been devised for the analysis of the relation between reaction rate and temperature (21, 22, 23). The result of the application of two of these to our data are given in figure 2. Both curves were fitted by the method of least squares. Curve A represents the logarithm of oxygen consumption plotted as a function of temperature in degrees Centigrade. The coefficient of correlation between the two variables was +0.995. The equation for curve A is:

$$\text{log oxygen consumption} = 0.286 + 0.0412\ t$$

where oxygen consumption is expressed in μl., N.P.T., per ml. enzyme per hour and t = temperature in degrees Centigrade. This is an application of the

Berthelot formula (Bělehrádek, 22, p 11) From the above equation the value of the van't Hoff temperature coefficient, Q_{10}, given by the slope, is antilog 0 412 or 2 58 While the correlation found here was good, there appears to be a slight but systematic deviation from the linear relationship in curve A in the range 15° to 35°C However, when the logarithm of the oxygen consumption was plotted as a function of the reciprocal of the absolute temperature a straight line was found to fit the data very well (curve B, fig 2) While these coefficients of correlation are not significantly different, the more consistent relation in curve 2 B appears to make this method of analysis preferable It follows that the relationship between oxygen consumption and temperature for the oxidation of
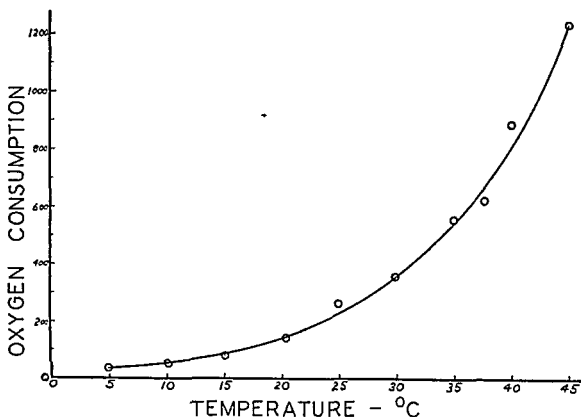
Fig 1 Curve Showing Oxygen Consumption of Amine Oxidase with Epinephrine Substrate as a Function of Temperature in Degrees Centigrade
Oxygen consumption expressed as µl per ml enzyme per hour

epinephrine by amine oxidase conforms to the Arrhenius equation (cf Bělehrádek, 22, Sizer, 24)

$$\ln \frac{K_2}{K_1} = \frac{\mu}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

in which K_1 and K_2 are the rates of oxygen uptake at absolute temperatures T_1 and T_2 respectively, R is the gas constant and μ is a constant called the thermal increment or temperature characteristic which has the meaning of a temperature coefficient (Bělehrádek, 22 p 16) For the oxidation of epinephrine, by amine oxidase at graded temperatures the value of μ, calculated from equation (2),

was 16,618. This is close to the values of μ found in a number of other enzymic reactions (cf. Sizer, 24, Table II).
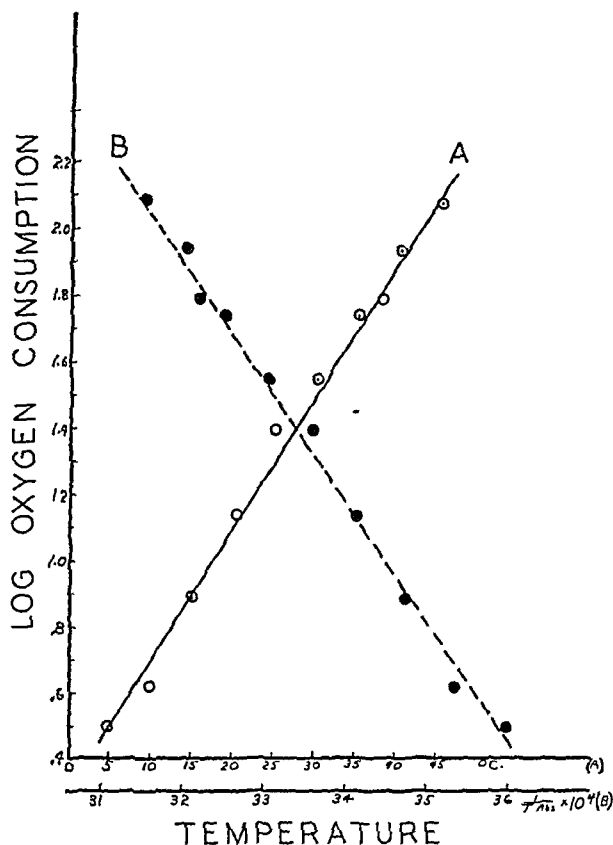
FIG. 2. A. Log oxygen consumption of amine oxidase with epinephrine substrate as a function of temperature in degrees Centigrade. B. Log oxygen consumption of amine oxidase with epinephrine substrate as a function of the reciprocal of the absolute temperature. Lines drawn were fitted to the data by the method of least squares.

PART 2. THE EFFECT OF TEMPERATURE ON THE RESPONSE OF THE NICTITATING MEMBRANE OF THE CAT TO EPINEPHRINE

It was shown in Part 1 that the oxidation of epinephrine by amine oxidase proceeds more slowly at reduced temperatures. If this oxidation or a similar mechanism is important in the inactivation of the hormone *in vivo*, it would be expected that in the hypothermic animal injected epinephrine would have either greater or more persistent effect (or both). To test this, the effect of injected epinephrine on the contraction of the acutely denervated nictitating membrane of the cat was determined at rectal temperatures ranging from 12.9° to 42.3°C.

METHODS   Cats were anesthetized with pentobarbital, the superior cervical ganglion excised on one side, and the nictitating membrane arranged for the recording of isotonic contractions   The animals were cooled by packing them in ice and wetting the fur when necessary   Rectal temperature was measured by means of a mercury thermometer inserted to a depth of about seven centimeters   In some animals regional warming was accomplished by long wave diathermy   For this purpose the electrodes, approximately 4 x 10 cm in size, were applied with electrode paste to shaved areas of the skin over the region of the liver, secured in place by adhesive tape and covered with rubber dam as a protection against wetting from the ice packs   The temperature in the warmed region was determined by a thermocouple inserted through a midline incision to a point near the hilus of the liver   The epinephrine used was 1 1000 "adrenaline chloride,' (Parke, Davis), diluted 1 5 or 1 10 and stabilized with blood   In three animals 0 2 ml of the solution was injected into a rubber tube connecting a cannula in the femoral vein with a burette containing Ringer's solution, and was washed in with 5 ml of fluid   In the other experiments injections of 0 1 or 0 2 ml , completed within five seconds, were made into the common carotid on the same side as the nictitating membrane being tested

RESULTS   (1) *The effect of epinephrine on the contraction of the nictitating membrane at varied temperature levels*   In general it was found that for a given dose of epinephrine the response of the nictitating membrane became progressively greater as the body temperature was reduced   The results on five animals are given in table 1   In these experiments the dose of epinephrine was not standardized on a unit weight basis   Instead, a dose of the drug which produced a small but definite response at about 36°C (approximate initial body temperature) was determined empirically for each animal   This dose was repeated at intervals as the body temperature was reduced   The doses used were small, ranging from 0 24 to 0 84 micrograms per kilogram   Three criteria of epinephrine effect on the nictitating membrane were used in the analysis of the thermal effects, namely, height of contraction (as recorded), duration of contraction and area under the response curve   Evidence bearing on the worth of these criteria under the conditions of our experiments will now be considered

(a) *Height of contraction*   Rosenblueth (25) reported that the curve showing the height of contraction of the nictitating membrane of the cat as a function of the dose of epinephrine was a rectangular hyperbola, and that the epinephrine concentration of unknown solutions could be estimated from this curve   Clark and Raventos (26) confirmed this for the nictitating membrane and also worked out dose-response relationships of arterial pressure and gut contraction   These observations were made at normal temperature levels   It is shown in Table 1 that the height of contraction of the nictitating membrane increased as the temperature was reduced, but that there was considerable irregularity in this temperature-response relationship, especially in the lower ranges of body temperature (20° to 26°C)

At about this level of hypothermia a critical fall in arterial pressure occurs in the dog and cat (27) and in the rat (28)   It is therefore probable that in this temperature range the circulation in the cat was impaired to such an extent that the concentration of epinephrine around the nictitating membrane did not reach the levels predicted on the basis of results at higher temperatures where the circulation was not impaired

Furthermore, the injected epinephrine may itself contribute to the circulatory impairment. Since enzymic breakdown of epinephrine is delayed by cold (Part I), the concentration of the hormone around the heart may become high enough to evoke fibrillation, although the same dose would not cause fibrillation at higher temperatures. To investigate this point, the electrocardiogram, visualized by a Sanborn cardioscope, was followed continuously in two animals. As body temperature fell, the effect of a small, constant dose of epinephrine in-

TABLE 1

Effect of temperature on the response of the cat's nictitating membrane to injection of epinephrine

ANIMAL	MEAN RECTAL TEMP.	DURATION	RECORDED HEIGHT	AREA
	°C.	sec.	mm.	sq. in.
Cat 1. Intravenous 0.2 cc. 1:10,000, 2.87 kg.	33.8	172	3	0.18
	33.8	148	3	0.17
	29.9	129	10	0.30
	24.7	336	18	1.47
	20.4	404	17	1.70
	18.6	694	17	2.53
	17.0	944	20	3.20
	13.6	1800	11	4.67
Cat 2. Intra-arterial 0.1 cc. 1:10,000, 4.21 kg.	35.0	108	5.5	0.09
	34.8	101	5.5	0.09
	29.5	379	19.0	0.92
	24.7	258	28.5	1.52
Cat 3. Intra-arterial 0.2 cc. 1:10,000, 2.72 kg.	37.8	20	1.5	0.02
	31.5	320	23.5	1.24
	26.1	516	26.5	2.60
	20.9	1155	34.5	9.92
	15.2	2526	26.0	10.36
	12.9	1892	35.0	6.71
Cat 4. Intra-arterial 0.1 cc. 1:20,000, 2.23 kg.	34.8	57	1.5	0.06
	27.9	442	15.0	0.84
	22.7	1076	28.0	4.05
	17.7	1614	13.0	5.21
Cat 9. Intra-arterial 0.1 cc. 1:10,000, 1.94 kg.	38.1	83	7.0	0.14
	40.6	92	5.0	0.09
	42.7	56	2.5	0.03

jected from time to time closely resembled the effects described by Milles and Smith (29) for increasing doses at normal body temperature. For example, in one of these animals at a rectal temperature of 24.7°, the injection of 0.1 ml. 1:10,000 epinephrine into the carotid artery produced no evidence of ventricular fibrillation and caused a 28.5 mm. excursion on the record of contraction of the nictitating membrane. At 20° the injection of the same dose produced fibrillation in a few seconds. There was no response of the nictitating membrane until

the fibrillation ceased and normal cardiac rhythm (for that temperature) was re-established, at which time a 6 mm contraction occurred   The same sequence of events was repeated after another injection at 19°C

It was concluded that the height of contraction of the nictitating membrane is not a satisfactory criterion of the rate of inactivation of epinephrine at these levels of hypothermia since impaired circulation complicates the interpretation of the results

(b) *Duration of contraction and area under response curve*   When either duration of contraction or area under the response curve was plotted as a function of temperature the dose-response relationship appeared to be an hyperbola   It follows that either logarithm of the duration or logarithm of the area should be approximately a linear function of temperature   In figure 3 the logarithm of the duration of the response (in seconds) is plotted as a function of temperature All available data on nine animals were used   The coefficient of correlation between the logarithm of time and temperature was 0 899, and the data were well fitted by a straight line   The equation of the regression line given in figure 3 is

$$\log \text{time (seconds)} = 4\ 042 - 0\ 0547\ t$$

where $t =$ temperature Centigrade

When the data for area under the response curve were treated in a similar manner the coefficient of correlation was lower, 0 715, and there appeared to be some deviation from a linear relationship, especially in the higher temperature ranges   It therefore appears that under the conditions of our experiments the duration of contraction of the nictitating membrane was the best measure of the rate of inactivation of epinephrine, and that this process is best depicted by figure 3

(2) *Effect of warming the region of the liver on the rate of inactivation of epinephrine in the hypothermic cat*   There is much evidence that the liver is the chief site of inactivation of epinephrine *in vivo*   Perfusion of the liver with solutions containing epinephrine resulted in marked reduction in concentration of the hormone in the perfusate (30, 31)   Injection of epinephrine into the portal vein produced much less pressor response than injection into the jugular vein (14, 17, 19, 32, 33)   Philpott and Cantoni (14) found that methylene blue inhibited the destruction of epinephrine by liver *in vitro*, and that the injection of methylene blue greatly increased the pressor response to the injection of epinephrine into the portal vein   Following hepatectomy the response to epinephrine administration is somewhat prolonged (34, 35)   Of the several enzymes possibly concerned in the inactivation of epinephrine *in vivo*, amine oxidase (7 8 36) and   sulfosynthase" (10 12) have been reported to occur chiefly in the liver

If epinephrine inactivation occurs mainly in the liver or even in the liver and surrounding viscera the duration of the response to epinephrine in the hypothermic cat would probably be shortened by local warming in the region of the liver   Such warming was effected with long wave diathermy as described under methods   The results are presented in table 2

It is shown in table 2 that a temperature differential of 2.1° to 5.9°C. was obtained between the liver and the rectum, and that under these conditions the duration of the contraction of the nictitating membrane on injection of epinephrine was decreased in every case.   The amplitude of contraction and the area under the response curve were also reduced.   Several measurements of the
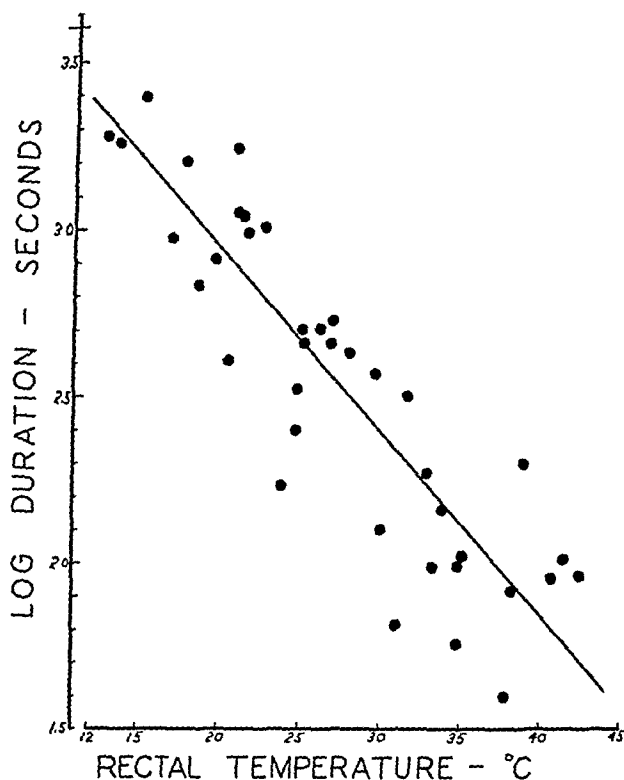
Fig. 3. Log Duration of the Response of the Acutely Denervated Nictitating Membrane of the Cat to Epinephrine Administration as a Function of Temperature

Data from Tables 1 and 2.   Line drawn was fitted to the data by the method of least squares.

temperature near the attachment of the nictitating membrane showed that it was within one degree of rectal temperature.

The effect of the regional warming is most clearly seen in the data on cat No. 8 (table 2).   In this case the rectal temperature remained approximately constant at 26.8°C. while the liver temperature was raised to 29.0°C.   This was a difference of only 2.2° but there was a reduction of 24 per cent in the duration of contraction, 41 per cent in the height and 66 per cent in the area under the response curve as compared with the values obtained at the same rectal temperature be-

fore warming the liver   Tracings of kymograph records in this series of experiments made after the liver temperature had again fallen to the level of the rectal temperature are presented in figure 4   A tracing of a control experiment made

TABLE 2

Effect of local warming on the response of the cat's nictitating membrane to the injection of epinephrine at reduced body temperature

ANIMAL	EXP'T NO	MEAN RECTAL TEMP	MEAN LIVER TEMP	LIVER RECTAL TEMP DIFF	DURA TION	AMPLI TUDE	AREA
		°C	°C	°C	sec	mm	sq in
Cat 6 Intra arterial 0 1	1	31 0	31 0		68	3 0	0 08
cc 1 20 000, 2 72 kg	2	25 6	25 6		464	18 5	1 67
		Diathermy					
	3	24 8	27 9	3 1	215	6 0	0 54
	4	23 2	29 1	5 9	266	5 5	0 49
		Diathermy off					
	5	20 8	20 8		1840	12 0	5 32
Cat 8 Intra arterial 0 1	1	33 2	33 1		97	3 5	0 17
cc 1 20 000 2 38 kg	2	32 8	32 5		190	3 0	0 18
	3	26 8	26 4		468	8 5	0 91
		Diathermy					
	4	26 9	29 0	2 1	357	5 0	0 31
		Diathermy off					
	5	27 1	2⁻ 0		548	9 0	1 18
	6	21 6	21 6		1000	13 0	2 21
		Diathermy					
	7	23 0	25 9	2 9	638	10 0	1 10
		Diathermy off					
	8	21 3	20 3		1122	12 0	2 64
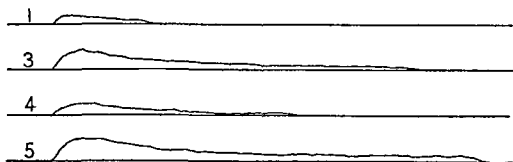
Fig 4 TRACINGS OF KYMOGRAPH RECORDS SHOWING THE EFFECT OF WARMING THE REGION OF THE LIVER ON THE RESPONSE OF THE ACUTELY DENERVATED NICTITATING MEMBRANE OF THE HYPOTHERMIC CAT TO ADMINISTRATION OF A GIVEN DOSE OF EPINEPHRINE

(1) Rectal temperature 33 2°C   Liver temperature 33 1°C (initial control experiment)
(3) Rectal temperature 26 8°C   Liver temperature 26 4°C
(4) Rectal temperature 26 9°C   Liver temperature 29 0°C (raised by diathermy)
(5) Rectal temperature 27 1°C   Liver temperature 27 0°C (final control experiment)

after the liver temperature had again fallen to the level of the rectal temperature is included   Clearly the relationships obtained are essentially the same as in the record just preceding that in which the liver was warmed

It should be pointed out that although the liver was warmed in these experiments the surrounding viscera must also have been warmed to some extent. Increased destruction of the epinephrine by other organs (especially the kidneys) may thus contribute to the increased inactivation which was observed.

DISCUSSION. It is shown in Part I that for at least one possible mechanism of epinephrine inactivation, oxidative deamination by amine oxidase, reduction of body temperature would lead to a greatly slowed rate of destruction. While this enzymic reaction is not the only, and perhaps not even the chief, route of epinephrine inactivation *in vivo*, it is clear that such inactivation is an enzymic process (26). Thus it is to be expected that reduction in temperature would markedly decrease the rate of the reaction involved (cf. Sizer, 24). It follows that the responses to the injection of epinephrine should be prolonged and intensified in the hypothermic animal. It is shown in Part II that this expectation is realized.

The effect of lowered temperatures on the muscle of the nictitating membrane itself must be considered in the interpretation of these results. The tone of smooth muscle is increased by cooling (37) and the duration of relaxation is prolonged (38). Elliott (19) found that the duration of contraction of the nictitating membrane (electrical stimulation), was prolonged from 30 seconds to 75 seconds by cooling. He concluded that the prolongation of the response to epinephrine in the hypothermic cat was too great to be explicable on the basis of effect of temperature on muscle. Stewart (38) found that when the smooth muscle of the cat's bladder was stimulated by single induction shocks at varied temperatures the duration of response at 15° was about 1.75 times that at 35°C., but that the amplitude was decreased at the lower temperature. While greater relative changes have been observed with frog smooth muscle on lowering the temperature (39), the findings on the cat, described above, are more relevant to the problem in hand. If these data be considered representative, it is clear that the prolongation of the response of the nictitating membrane in our hypothermic cats (Table 1) is much too great to be explained on this basis. For example, in cat No. 3 the maximum duration observed (at 15.2°) was 126 times the minimum duration (at 37.8°). Further evidence for the view that slowing of the rate of inactivation was the chief cause of the increased response of the nictitating membrane to epinephrine at lowered body temperatures is afforded by the experiments on regional warming. In these the duration of response was greatly reduced by warming only the region of the liver, with little change in either rectal temperature or in the temperature of the nictitating membrane.

The effect of hypothermia in increasing the intensity and duration of the action of epinephrine may be of importance in relation to chemical regulation of body temperature. Cannon and his associates (40) have demonstrated the importance of epinephrine secretion in the cold defense responses of the cat, and lowered body temperature would intensify the effects of increased secretion of the hormone. In the rat the role of epinephrine in cold defense is not so clear (41, 42).

The increased response to epinephrine in the hypothermic animal may be of

clinical significance While the slowed heart and lowered temperature of the hypothermic patient might suggest the use of epinephrine, there are at least two reasons for unusual caution in the use of this drug under these circumstances These are the increased likelihood of cardiac failure and of failure of the peripheral circulation Thus doses of epinephrine which produce little effect at normal body temperature may result in toxic reactions such as heart block or fibrillation in the cooled body because of delayed inactivation Secondly, vasoconstriction may already be excessive, so that administration of epinephrine, evoking further vasoconstriction, might lead to circulatory failure due to anoxia of peripheral tissues with consequent increase in capillary permeability and loss of plasma to the tissues (43, 44)

SUMMARY

1 The rate of oxidation of epinephrine by amine oxidase *in vitro* at graded temperatures can be described by the Arrhenius equation The value of the thermal increment (μ) for this reaction was 16,618 over the range 5° to 45°C

2 The effect of injected epinephrine on the contraction of the acutely denervated nictitating membrane of the anesthetized cat was determined at rectal temperatures ranging from 12 9° to 42 3°C

3 Evidence was presented which indicated that the duration of contraction of the nictitating membrane was a more satisfactory criterion of the rate of inactivation of epinephrine in the hypothermic cat than either the amplitude of contraction or the area under the response curve

4 For a given dose of epinephrine the response of the nictitating membrane became progressively greater as body temperature was reduced This was in harmony with the observations on the oxidation of epinephrine by amine oxidase at graded temperatures

5 Regional warming of the liver (long wave diathermy) of hypothermic cats without change in either rectal temperature or in the temperature of the nictitating membrane shortened the response of the membrane to a given dose of epinephrine

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THE SPASMOLYTIC AND LOCAL ANESTHETIC ACTION OF SOME ESTERS OF 9,10 DIHYDROANTHRACENE-CARBOXYLIC ACID AND RELATED COMPOUNDS[1]

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In a previous paper (1) it was shown that the introduction of a carbon-carbon bridge between the two phenyl groups of some diphenylacetic acid esters led to new compounds of greater spasmolytic potency But only the relaxing power against acetylcholine induced spasm was augmented whereas little or no change had occurred in their spasmolytic activity against histamine induced spasm In this respect none of the compounds studied previously was greatly superior to papaverine, with only one exception Replacement of the carbon carbon bridge by a methylene group leads to the corresponding dihydroanthracene carboxylic acid ester, a compound with marked anti-histamine activity The spasmolytic and local anesthetic activity of this compound and of others obtained by exchanging the methylene group for other groups with similar electronic configuration as O, S, NH (fig 1), have been studied The compounds used in this investigation have been synthesized by R R Burtner and J W Cusic (2) at G D Searle & Co, Chicago, Ill

EXPERIMENTAL   Spasmolytic activity of all compounds was determined on isolated intestinal strips of rabbits and guinea pigs against spasm induced by acetyl choline bromide (10^{-6} gm /cc), histamine phosphate (2×10^{-6} gm /cc) and barium chloride (10^{-4} gm /cc) as described elsewhere (1) The reciprocal activity ratios with reference to diethylaminoethylfluorene-9 carboxylate (T1) *as arbitrary standard, depth and duration of local anesthesia on the rabbit's* cornea and the figures for intraperitoneal toxicity in mice are shown in table 1 For details of determination we refer to a previous paper (1)

The spasmolytic potency of some compounds of this series has been studied ' in vivo" on the rabbit's ileum Fifty-eight injections were made into seven rabbits in urethane anesthesia with the addition of some ether in most cases The spasmolytic activity ratios gained from these experiments are in good agreement with those obtained on the isolated rabbit's intestine against spasm produced by acetyl choline The results are summarized in table 2

Two compounds of the series deserved further study, compound no 1 for its powerful anti histamine action and compound no 9 because of its great all around spasmolytic potency

The antagonistic action of compound no 9 against the vaso depressor effect of acetyl choline was determined in the etherized dog according to Kuhl (3)

It was found to be nearly twice as active as F1 and seventy times weaker than atropine. In its mydriatic action it is more than twice as potent as F1, but about one thousand times weaker than atropine (table 3). The antisialogogue action after pilocarpine is more than one hundred times weaker than that of atropine, but slightly stronger than that of F1 (fig. 2).

The strong histamine antagonizing action of compound no. 1 is not limited to the guinea pig's ileum. It is capable of preventing death from histamine in guinea pigs. Six unanesthetized guinea pigs received 50 milligrams per kilogram of compound no. 1 subcutaneously. Thirty-five minutes later 1.7 mgm./

Diphenylacetic acid

Fluorene carboxylic acid

Dihydroanthracene carboxylic acid

Xanthene carboxylic acid

Thioxanthene carboxylic acid

Acridan carboxylic acid
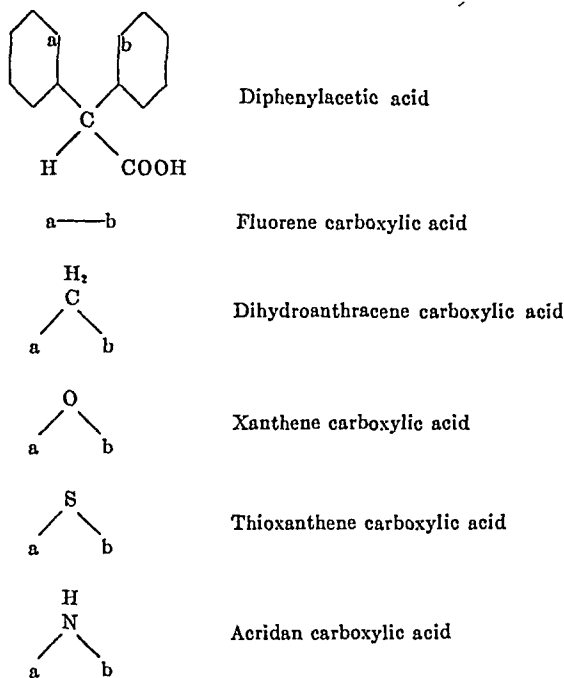
Fig. 1

kgm. histamine phosphate were injected intracardially. Three of the animals showed signs of moderate dyspnea, but all six survived. One hundred milligrams per kilogram of F1 given to a second group of six guinea pigs thirty-five minutes previous to the intracardial injection of 1.7 mgm./kgm. histamine phosphate did not have a protective effect. All animals died within three to seven minutes with the same symptoms of asphyxia as seen in a third group of six guinea pigs which succumbed following the intracardial injection of 1.7 mgm./kgm. histamine phosphate only.

When tested on the isolated perfused guinea pig's lung, according to Thornton

TABLE 1

NO	ACID	ALCOHOL	Acetyl choline	Histamine	Barium	Duration (min)	Depth	LD50	0.1	0.2	0.3	0.4	0.5	0.6	0.8	1.0
1	..droanthracene 9 carboxylic	β-Diethylaminoethanol	5	2	1	33	+++	0.15		0/6/6/6						
2	..droanthracene 9 carboxylic	γ-Diethylaminopropanol	5	2	0 5	32	+++	0.20		0/6/6						
3	..droanthracene 9 carboxylic	β-Diethylaminopropanol	10	2	10 0 5	26	+++	0.21			6/6					
4	..droanthracene 9 carboxylic	β-Di-n-butylaminoethanol	100	25	2	25	+++	0.53	1/6							
5	..droanthracene-9 carboxylic	β-Morpholinoethanol	50	2	1	14	+++	0.50				1/6		4/6/6		
6	..droanthracene-9 carboxylic	(bis) β-Diethylaminoethanol	30	1	0 5	13	++	0.50				1/6/3/6/6				
7	..dro-10 methylanthracene-ylic	β-Diethylaminoethanol	20	0 1	1	23	+++	0.17	17/6		6/6					
8	..dro 9-methylanthracene-9-lic	β-Diethylaminoethanol	5	0 1	1		+++	0.15		0/6/6						
9	..-9-carboxylic	β-Diethylaminoethanol	0 5	0 4	0 5	16	+++	0.25		1/12	10/12					
10	..hene-10-carboxylic	β-Diethylaminoethanol	6	0 33	2	27	+++	0.22		0/3/6	6/6					
11	..l-carboxylic	β-Diethylaminoethanol	3	1	1	14	++	0.14		0/6/6						
12	..lcridan-9-carboxylic	β-Diethylaminoethanol	40	1	5	23	+++	0.15		0/6/6						
13	..norene-9 carboxylic	β-Diethylaminoethanol	10	0 5	33	32	+++	0.15		0/6/6						
14	..xylfluorene-9-carboxylic	β-Diethylaminoethanol	50	3	4	8	+	0.15		15/5/6						
15	..carboxylic	β-Diethylaminoethanol	100	3	5	0	0	0.23		0/3/2/6						
16	..luro-1-naphthou	β-Diethylaminoethanol	60	3	3		+	0.60		0/6/2/6	5/6					
17	Phenanthrene-9-carboxylic	β-Diethylaminoethanol	50	2	3	38	+++	0.24		0/6/4/6	5/6		2/6/5/6			
18	..ene-10-carboxylic	β-Diethylaminoethanol	40	4	3	60	+++	0.24		10/6/4/6	0/6					
19	Anthracene-10-carboxylic	γ-Diethylaminopropanol	200	30	30		0	0.17		0/6/6	0/6		2/6/6/6			5/6/6/6/6/6
20	Anthracene-10-carboxylic	β-Diethylaminopropanol	30	0 7	2	29	+++	0.43			2/6		2/6/6			
21	Phenyl, cyclohexyl-glycollic	β-Di-n-butylaminoethanol	0 85	0 14	20		0	0.47		0/6	4/6					
22	Atropine	β-Diethylaminoethanol	0 14	1	1		0	0.27	0/6	0/6/1/6	6/6					
23	Papaverine		30	1	5		+++	0.25								

All compounds except 22 as hydrochlorides, 22 as sulfate

Mortality ratios = dead animals/animals used

TABLE 2

Comparison of relaxing effect on rabbit's ileum in situ

$$\#1 < \text{F1}^*$$
$$5 \times \#1 = \text{F1}$$
$$\#1 = 4 \times \#7$$
$$\#9 = 3 \times \text{F1}$$
$$\#9 > \#11$$
$$10 \times \#14 = \text{F1}$$
$$\#22 > 3 \times \text{F1}$$

* Diethylaminoethyl fluorene-9-carboxylate

TABLE 3

Pupil size in millimeters

HOURS	LEFT EYE	RIGHT EYE	LEFT EYE	RIGHT EYE	LEFT EYE	RIGHT EYE	LEFT EYE	RIGHT EYE
0	Atropine sulfate 0.001% in left eye		Xanthene derivative 5% in left eye		Xanthene derivative 1% in left eye		Fluorene derivative 2% in left eye	
0.5	3	2	9	2	7	2	5	2
1.0	3	2	9	2	7	2	6	2
1.5	5	2	9	3	6	3	5	2
2.0	5	2	9	2	5	3	4	2

The results of four separate experiments on the same cat on different days.
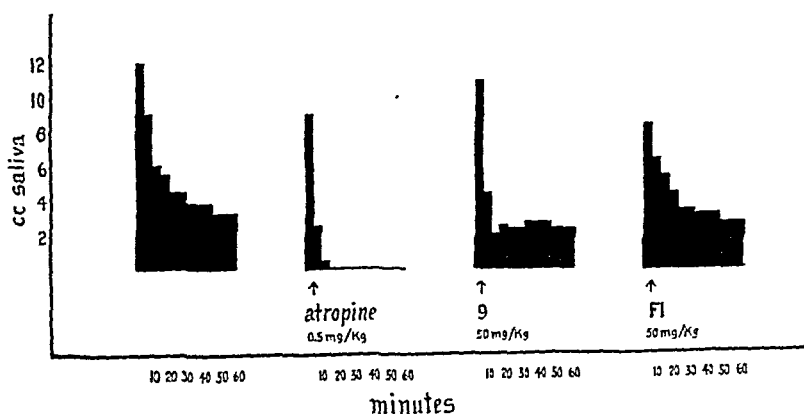
FIG. 2. SECRETION OF SALIVA

Four rabbits, urethane 1 gm. per kgm. per os; pilocarpine 15 mgm. per kgm. subcutaneously in each.

Ordinate: saliva in cc.

Abscissa: minutes.

From left to right: control, at arrows: subcutaneous injection of atropine 0.5 mgm. per kgm., diethylaminoethyl xanthene-9-carboxylate 50 mgm. per kgm. and diethylaminoethyl fluorene-9-carboxylate 50 mgm. per kgm.

(4), much smaller amounts of compound no. 1 than of F1 were required to produce bronchodilation.
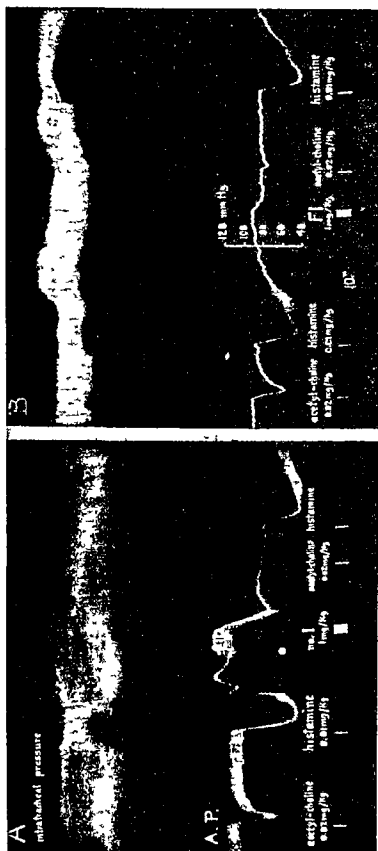
FIG 3 DOG, BARBITAL ANESTHESIA, THORAX OPENED, PHRENICS AND VAGI CUT, POSITIVE PRESSURE ARTIFICIAL RESPIRATION INJECTIONS INTO FEMORAL VEIN

From the top down, intratracheal pressure, carotid arterial pressure, signal of injection, time in ten seconds
A From left to right acetyl-choline 0 02 mgm per kgm, histamine 0 01 mgm per kgm, diethylaminoethyl 9,10 dihydroanthracene 9 carboxylate 1 mgm per kgm, acetyl choline 0 02 mgm per kgm, histamine 0 01 mgm per kgm
B From left to right, acetyl choline 0 02 mgm per kgm, histamine 0 01 mgm per kgm, diethylaminoethylfluorene-9 carboxylate 1 mgm per kgm, acetyl choline 0 02 mgm per kgm, histamine 0 01 mgm per kgm
Between A and B, thirty minutes

Increasing the number of carbon atoms in the aminoalcohol decreases spasmolytic potency to a much greater extent than toxicity, as has been previously observed (1).

There is no obvious relationship between spasmolytic and local anesthetic activity.

SUMMARY

The spasmolytic and local anesthetic action and the toxicity of a series of twenty-one basic esters of polynuclear carboxylic acids have been studied.

The diethylaminoethyl ester of xanthene-9-carboxylic acid has the greatest average spasmolytic activity.

The diethylaminoethyl ester of 9,10-dihydroanthracene-9-carboxylic acid is outstanding for its powerful histamine antagonizing action on smooth muscle. This has been demonstrated on the ileum, on the bronchioles, and to a lesser degree on the blood vessels.

This compound also antagonizes the effect of acetyl-choline and barium on the ileum, and the effect of epinephrine on the uterus, the nictitating membrane, and the blood vessels.

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STUDIES ON THE RELATION OF DRUG ADDICTION TO THE AUTONOMIC NERVOUS SYSTEM RESULTS OF TESTS OF PERIPHERAL BLOOD FLOW[1]

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It is well known that the effect of morphine has definite appeal to certain maladjusted persons. It gives them a sense of well being. The reason for this effect is not yet known, but it is suggested that morphine accomplishes it through reducing the reactivity of the sympathetic (thoracolumbar) nervous system, thus interfering with vicious psycho somatic cycles entailed in such maladjustments. At any rate in such persons the resulting temporary euphoria motivates repetition and this in time inculcates a strong emotional attachment to the drug. Unfortunately for the user, the basic cause of the maladjustment is not eliminated, in fact additional problems such as tolerance, expense, law violation, loss of self respect, ostracization, and physical dependence are brought to bear on an already unstable nervous system.

This view presupposes that addiction proneness generally is closely related to instability of the autonomic nervous system. Since the disturbing features of the *external* environment generally credited as causal in the etiology of addiction and relapse do not appear to be disproportionate to ordinary life situations it seems appropriate to consider the possibility of a defective *internal* environment, and to extend studies of the functional integrity of the autonomic nervous system of addicts and post addicts.

In a previous report (1) it was shown that the pressor response to cold in stabilized morphine addicts is greater than normal, and that recovery is slower than normal. Since the acute effect of morphine is the converse, it was suggested that physical dependence might constitute, in part, a state of hyperirritability of the sympathetic nervous system developed to maintain homeostasis in the continued presence of an effect disturbing to equilibrium. The possibility that morphine has a peripheral vasoconstrictor effect to which little or no tolerance is developed in contrast to its central nervous system action, was not mentioned. Since this hyper reactor' response of addicts reverted slowly to normal in about 6 months after withdrawal, it seemed that if the etiology of addiction and relapse is associated with autonomic dysfunction a more sensitive method would be required to detect it.

Terry and Abramson (2) have indicated the usefulness of the plethysmographic technique in studying blood flow to extremities and the physiologic significance of such studies. It appears that the control of blood flow to the hand is dominated by the sympathetic nervous system and subserves chiefly the regulation

of body temperature (3, 4) whereas blood flow to the forearm (except for the skin) subserves chiefly trophic functions (2). Thus, study of blood·flow to the hand by the plethysmographic technique seemed to offer a method for further investigation of the influence of morphine and morphine addiction on the sympathetic division of the autonomic nervous system.

METHODS. *Technique.* The rate of blood flow to a portion of the upper extremity was measured by the venous occlusion plethysmographic method of Hewlett and Van Zwalu-wenburg (5). The plethysmographs and accessories used were constructed as described by Ferris and Abramson (6) except that changes in volume were recorded on moving paper from water manometer floats.[2]

The majority of these studies were made in a sound-shielded, temperature and humidity controlled room (78°F., relative humidity 50 per cent). In the remainder the dry bulb room temperature range was 77°–82°F. Subjects were in the room for at least two hours before the test. A low calorie, high carbohydrate, low protein, low fat meal was served two to three hours before the test (7). No smoking was permitted from at least one-half hour before until completion of the test (8). Other physiologic and environmental factors which might influence the validity of the results were reduced to a minimum (9, 10, 11). All tests were made with subjects in the recumbent position with the extremity under study at or slightly above the level of the right atrium. A bath temperature of 32°C. (±2) was used in most of the experiments. Calibrations were made by injecting known amounts of water into the plethysmographs before and at the conclusion of tests. A cuff pressure of 70–80 mm. Hg was maintained during the process of calibration.

Studies were made either on both hands simultaneously, or on the left forearm. A sub-diastolic pressure of 70 mm. Hg (±5) was used for venous occlusion. When studying the forearm, a pressure of 300 mm. Hg was applied at the wrist just distal to the plethysmograph (12). Blood pressure, pulse rate, oral temperature, and bath temperatures were recorded at appropriate intervals.

After obtaining 20 to 35 satisfactory records of the resting blood flow, the medication was administered subcutaneously in the pectoral region. When studying the hand 10 to 20 flows were recorded $\frac{1}{4}$, $\frac{1}{2}$, $\frac{3}{4}$ and 1 hour after the dose. The average blood flows in cc. per 100 cc. of extremity per minute were calculated for each period. When studying the forearm, after recording 20 to 30 resting flows, an exercise test was administered. Fifteen to twenty-five resting flows were recorded 20 to 30 minutes and 50 to 60 minutes after the medication, then the exercise test was repeated. The exercise consisted of having the subject inflate a 5 gallon bottle to 70 mm. Hg pressure by compressing a sphygmomanometer bulb with the ipsilateral hand (13). Post-exercise records of blood flow were made at frequent intervals for the ensuing 15 minutes and the mean blood flow in each minute was calculated. The additional flow of blood to the forearm in the 15 minutes immediately after the exercise was calculated from the area of a graph constructed from these points with the pre-exercise resting values as base lines. *Subjects:* The subjects used in these studies were addicts, post-addicts, and controls; all were free of complicating conditions. The addicts were patients with valid physical dependence, stabilized on amounts of morphine just sufficient to prevent abstinence phenomena. The post-addicts were prisoner patients who had been morphine addicts and in whom withdrawal had been accomplished at least 6 months previously. Where the same post-addict was studied on two or more occasions, the interval between tests was at least two weeks. Two groups of controls were employed; one consisted of civilian hospital personnel and the other of incarcerated marihuana users who gave no history of opiate addiction.

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[2] The recording system used in these studies will be described in detail elsewhere by Andrews, H. L. and Himmelsbach, C. K.

Blood flow to the hands was studied in two post addicts prior to and during a six months period when morphine was administered practically ad libitum

Studies were made on the effects of 10, 20 30, and 40 mgm morphine sulfate on the blood flow to the hands of a patient who had sympathetic paralysis of the right upper extremity This patient had complete anhydrosis on the affected side, and his right hand generally seemed warmer than the left

RESULTS   *Effect of morphine on blood flow*   Morphine increased the rate of blood flow to the hands in addicts, post addicts, and normal controls   In the few instances where continuous post dose records were made, this effect became noticeable within 5 to 10 minutes   With all doses except 5 mgm the increase was statistically significant within $\frac{1}{4}$ hour   The results in detail are presented in table 1   Demerol likewise caused a significant increase in the blood flow to the hands   Very slight and non significant changes occurred following the sub cutaneous injection of one half cc of normal saline

The average results obtained with 20 and 40 mgm morphine, and $\frac{1}{2}$ cc normal saline on the hand, and 30 mgm morphine on the forearm are presented graphically in figure 1   Since the results with normal saline indicate the feasibility of using the pre injection (resting) values as base lines, the data were plotted in this manner, and the total change in one hour calculated from measurements of the enclosed areas with a planimeter   The total effects of the various doses of morphine for the first post injection hour are shown in figure 2

A small, but nonetheless statistically significant increase in blood flow to the forearm resulted from the administration of 30 mgm morphine sulfate   The exercise tests yielded the following result   The difference in the amount of additional blood flow before and one hour after morphine, was equal to the difference accountable by the post-dose increase in resting blood flow (fig 3)

Average concomitant effects of morphine   A progressive but small (less than 1°C) increase in hand *bath* temperature occurred in all instances where morphine or Demerol was given   Less than 0 1°C increase followed the administration of normal saline   A slight decrease (0 3°C) occurred in forearm *bath* temperature following morphine   A progressive, small (0 2°–0 3°C) fall in *oral* temperature occurred in all instances where morphine or Demerol was given, but only 0 1°C decrease followed normal saline

A minor decrease in pulse rate (1–7/min) generally, but not regularly, followed administration of morphine or Demerol   The pulse rates during the normal saline tests were stable   No significant or consistent changes were noted in either systolic or diastolic blood pressure   Particular attention was not paid to alterations in hand volumes, "spontaneous" or otherwise, or in venous capacity   In about one half of the experiments a volume increase was noticed after morphine, sometimes a decrease was observed

Resting blood flow   A comparison of the mean data on resting blood flow to the hands of post addicts, addicts, normal controls, and marihuana (non opiate) users is shown in table 2   The critical ratio of the difference between the normal values for blood flow to the hand reported by Abramson and Fierst (14) and this group is 0 56   The values for post addicts and addicts were found to be significantly lower than normal, but that of marihuana users was normal

Studies of blood flow to the hands of two subjects who were given morphine practically ad libitum for six months showed some reduction to accompany the reproduction of addiction.

Repeated tests on individual post-addicts failed to show any trend to result from practice.
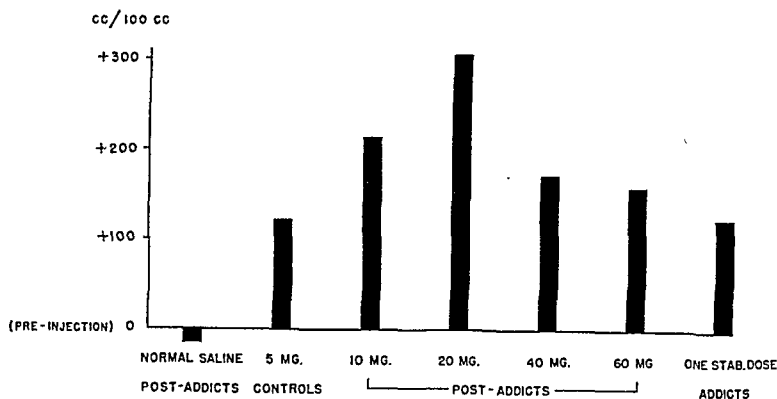
FIG. 2. TOTAL EFFECT OF MORPHINE ON BLOOD FLOW TO THE HAND IN ONE HOUR
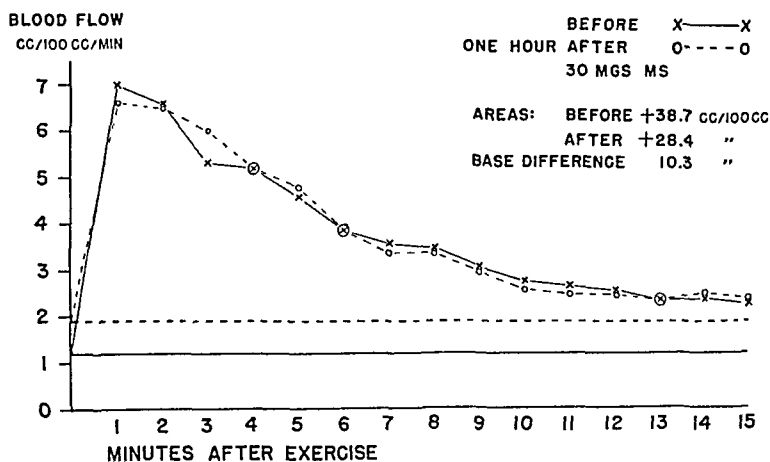
FIG. 3. INFLUENCE OF MORPHINE ON RESPONSE TO EXERCISE

Effect of morphine on blood flow to the hands of a patient with unilateral sympathetic paralysis. In one patient with sympathetic paralysis in the right upper extremity 20, 30, and 40 mgm. morphine failed to increase the blood flow to the affected hand, but had the usual effect on the contralateral hand. Blood flow to the affected hand was reduced slightly (maximum 11 and 18 per cent) following 40 and 20 mgm. morphine. Ten mgm. had no effect on the right hand, and only

slight effect on the left   On several occasions it was observed that a painful pinch resulted in a definite reduction in volume of the left hand, while little or no effect occurred in the right hand (11)   "Spontaneous" volume changes were

TABLE 2

(a) *Blood flow to hand*

	NO	MEAN	σ(DIS)	σ(AV)
Normal (Abramson and Fierst) (14)	61	9 3	2 1	0 18
Normal (Himmelsbach)	22	9 5	3 2	0 5
Incarcerated Marihuana users	19	9 8	3 6	0 58
Post addicts (opiate)	51	8 1	3 5	0 25
Stabilized opiate addicts	17	6 3	2 4	0 47

(b) *Critical ratios of differences*

	(A)	(B)	(C)
(A) Normal (Himmelsbach)			
(B) Incarcerated Marihuana users	0 39		
(C) Post addicts (opiate)	2 5	2 7	
(D) Addicts (stabilized opiate)	4 6	4 7	3 4

TABLE 3

Plethysmography protocol

Name *S*——   No *7252*   Status *Pris*   Date *8-20-43*
Age *56*   Medication *Morphine sulfate*   Dose *30 mgm*   at *9 30 a m*
Entered at *6 45 a m*   Meal at *6 30 a m*   CHO *123 gm*   P *43 gm*   F *0 6 gm*
Smoked at *8 30 a m*   Sleep *5 hours fitful*
Volumes R(*480 cc*) L(*490 cc*) Calibrations R(*1 53 cm /cc*) L(*1 56 cm /cc*)

TIME	9 30	9 45	10 00	10 15	10 30
Room temperature w/d(°F)	65/78	65/78	66/ 8	66/78	65/78
Right					
Blood flow (m)	6 4	6 3	5 8	6 4	6 0
No of flows	30	20	19	20	21
Bath temp (°C)	31 2	31 2	31 5	31 8	31 7
Left					
Blood flow (m)	5 6	9 9	10 1	10 2	9 9
No of flows	30	20	19	20	21
Bath temp (°C)	30 7	30 8	31 2	31 8	32 0
Oral temperature (°C)	36 4	36 2	36 0	35 9	35 9
Pulse rate /min	56	56	60	56	56
B P mm Hg	146/90	142/86	144/88	156/94	156/90

Remarks B P same on both sides   Some pain and discomfort before, and some relief by medication   Right hand perceptibly warmer than left   Patient cooperative and quiet throughout   No remarkable volume changes noted

present on the left, absent on the right   The bath temperature on the right was usually quite stable, whereas that on the left increased after morphine

The protocol of the experiment with 30 mgm morphine is shown in table 3 and representative samples of the records are presented in figure 4

Supplementary observations. It is well known that cold will reduce and heat increase blood flow to the hand; the direct effects being usually greater than the indirect or reflex. The influence of morphine was studied on hands in which blood flow had been increased or decreased, both directly and reflexly, in several normal post-addicts and the patient with unilateral sympathetic paralysis. For
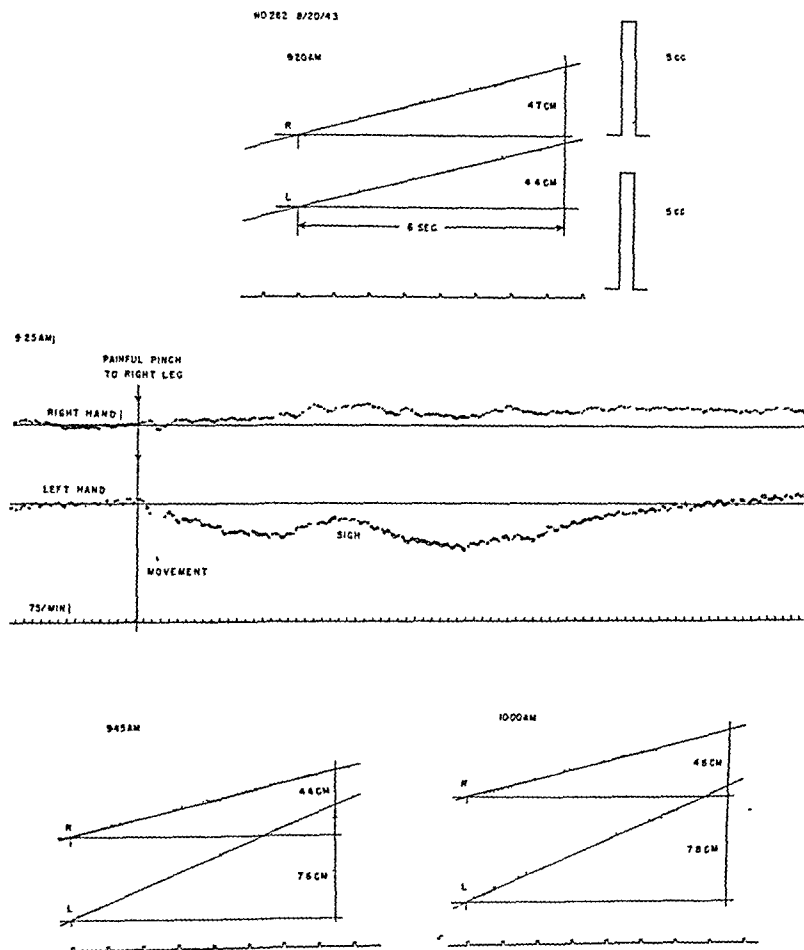
FIG. 4

these purposes the plethysmograph water temperature either was raised to 41–44° C. or lowered to 13–20°C. Local cold would reduce and local heat increase blood flow to the affected hand (right) of the patient with unilateral sympathetic paralysis. Reflex changes occurred contralaterally. When the situation was reversed however, blood flow to the right hand was not affected. Thus the right

hand did not respond reflexly to either heat or cold   Morphine resulted in a slight increase in blood flow to this patient's right hand after the flow had been increased by direct heat, but after the blood flow to the right hand had been decreased by direct cold, some further reduction occurred after morphine

Morphine had its usual effect on normal hands in which the flow had been altered reflexly, but its effect was reduced greatly after flow had been diminished by direct cold and somewhat when flow had been increased by direct heat

Other experiments on the patient with unilateral sympathetic paralysis revealed that premedication with neither benzedrine (10 mgm) nor prostigmine (1 mgm) would so influence the affected hand that it would respond to morphine However, flow to this hand was increased from 7 5 to 14 8 cc /100 cc /min after ingestion of 2 oz of 100 proof whiskey

The effect of morphine on relative intracranial blood flow was studied in a patient with a triangular skull defect (6 x 9 cm) in the right temporal region Following venous occlusion accomplished by inflating a neck cuff suddenly to 70 mm Hg the skin overlying the defect would bulge outward, the extruded mass increasing in size with each heart beat   Records were made by means of a tam bour cemented over this area   The rate of blood flow was increased greatly after 20 mgm of morphine

DISCUSSION   *Effect of morphine on blood flow*   The literature concerning the effect of morphine on the circulation has been reviewed thoroughly by Eddy (15) Morphine is considered to be depressant to the circulation

While attempts at localization of the action of a drug on the intact subject are hazardous, these results would tend to confirm the impression that morphine in some manner effects a reduction in sympathetic activity   Warren et al, (16) reported a great increase in blood flow to the hand of a subject in whom they produced unilateral sympathetic paralysis by novocaine block of the paravertebral ganglia   In the patient with unilateral sympathetic paralysis, the results suggest that an increase in blood flow to the hand after morphine depends on intact sympathetic control and that the peripheral effect results from central action of the drug   Since the blood flow to this patient's affected hand was altered significantly by direct heat and cold and after the ingestion of alcohol, but not after morphine, it seems unlikely that morphine (in the doses employed) has a physiologically significant direct effect on blood vessels   This finding is not in serious conflict with the results reported by Schmidt and Livingston (17) for they demonstrated very clearly that only the first *intravascular* dose of morphine administered to non tolerant cats or dogs has a direct effect on blood vessels dilator in those of skin and muscle, constrictor in those of the viscera   It is not possible to determine from these observations whether this central action of morphine mediated by the sympathetic nerves, is due to direct or indirect depression of sympathetic tone general depression of the central nervous system, or a more selective effect on hypothalamic thermo regulatory centers

The finding that morphine in doses of 40 and 60 mgm caused less overall effect than 10 and 20 mgm is of some interest   With the larger doses the initial effect was not dissimilar from that of the smaller, but was less well maintained

This might be interpreted to mean that as the effect of the larger doses approached their maxima, forces having to do with the maintenance of homeostasis became appreciably activated, i.e. counter-action was evoked. This view is consistent with the subjective estimates of a number of patients that these doses were uncomfortably large. On the other hand this finding might be interpreted as evidence of a delayed central stimulant action of morphine, or a delayed direct tonic effect on blood vessels, not apparent with the smaller doses.

An interpretation of the effect of morphine on blood flow to the forearm is somewhat more difficult since it is possible that the exercise per se was sufficient to cause a maximal response. Since the blood flow to the skin of the forearm is influenced by the sympathetic for purposes of temperature regulation, it would be consistent to estimate that the observed increase in blood flow after morphine was predominantly in the skin, not muscle.

Resting blood flow to the hand. Subnormal blood flow to the hands is considered to indicate increased sympathetic tone and to reflect a state of tension. The results are consistent with the view that post-addicts are more tense than normal. Since convicted marihuana users studied under the same conditions exhibited normal blood flow, it would appear that this manifestation of tension in post-addicts is a precursor to and/or a residuum of opiate addiction and not a product of incarceration.

The results on addicts are in accord with the view that nervous instability is greater after dependence is established than before, and that the chief effect of morphine then is to maintain psuedo-normal physiologic equilibrium (18). In two addicts, psychically satisfying amounts of morphine (1 to 2 gm. per day) did not appear to accomplish more than stabilizing amounts in other addicts.

The general implication of these results, so far as the addiction problem is concerned, would seem to be that the post-addict is tense; that morphine relieves this through depression of the sympathetic division of the nervous system; and that larger doses tend to evoke counter-action. An extension of this latter process might help explain the nature of physical dependence (19).

CONCLUSIONS

1. The resting blood flow to the hands of addicts and post-addicts is subnormal, while that of marihuana users is normal.

2. Morphine significantly increases the rate of blood flow to the hand and forearm. The total increase to the hand in the first post-injection hour is greater with 10 and 20 mgm. than with 40 and 60 mgm. The effect on the hand seems to require intact sympathetic nerve control.

3. Demerol increases blood flow to the hand.

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THE ACTION OF ATABRINE ON THE ELECTRO CORTICO POTENTIALS

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Various reports by clinical observers in Ceylon, India, Malaya describe certain mental effects during or following the administration of atabrine in therapeutic doses in malaria. Complications varying from mental depression, hyperexcitability and psychosis with temporary insanity were observed (1, 2, 3, 5, 12). Despite the low incidence of mental symptoms during or subsequent to the administration of atabrine noted in the Western Hemisphere (1), a most important question arising is whether these mental disorders following atabrine therapy result as complications of severe malaria infection or are due to the action of atabrine upon the central nervous system.

The following investigations were undertaken in order to clarify this question and to determine to what extent atabrine in *non-toxic* doses exercises an effect upon the central nervous system independent of the malarial infection. Previous experiments have demonstrated that only large, usually fatal doses produced cerebral stimulation accompanied by clonic and tonic convulsions and dyspnea, whereas with small doses gastro-intestinal symptoms were predominant (4, 5, 7). Since it is known that drugs affecting the central nervous system may cause a change in the electro-corticogram, we decided to employ this method as a convenient means of studying the reaction of the brain cortex to varying doses of atabrine. The drug is retained for some time in all the tissues including the brain (3, 5, 9, 10, 11); hence, changes occurring in the electro-potential of the cortex could be regarded as due to the central action of atabrine.

METHODS. Preliminary tests were undertaken on non-narcotized *pithed frogs* weighing 30–40 grams. Aqueous atabrine solutions were used since earlier investigations demonstrated that the electro-potentials of the brain of these animals are easily altered by various drugs. For this purpose three Hoagland electrodes were introduced through the skull. The first lead was recorded from the anterior portion of the left hemisphere, the second from the hind portion of the right hemisphere and the third lead from the left optic lobe. The leads were paired in three channels, the first from electrodes 1 and 2, the second from electrodes 2 and 3 and the third channel from electrodes 1 and 3. Bleeding was carefully avoided. Then 0.5 mg.–2 mg. of atabrine in aqueous solution was injected into the anterior lymph sac.

Doses of 0.5–1 mg. injected into the thoracic lymph sac of *normal frogs* caused no notable effects; there was only slightly increased reflex excitability and contraction of the melanophores. Twenty minutes following the injection of 2 mg. of atabrine, the frogs lost their righting reflexes, and remained lying on their backs; they jumped awkwardly when touched and performed crawling movements. Within one hour after the injection all extremities

were paralyzed The heart action was not affected The previously dark frogs became notably pale After several hours, the motor paralysis completely disappeared, and the pallor vanished Three milligrams produced a complete motor paralysis which like the paling of the animals persisted for days However, in most instances, 3 mgs was a fatal dose, paralyzing the whole motor activity and finally also the heart

Further tests were conducted on thirty *cats* ranging in weight from 1 75-3 kg Since frequent spontaneous movements of these animals interfered with the recording of the cortical impulses, the experiments were carried out under anesthesia Nembutal in doses of 30 mgm per kilogram was injected intraperitoneally Several of the animals vomited during the initial phase of the anesthesia, the majority, however, showed a fairly constant plane of anesthesia with reflexes absent and spontaneous regular respiration unaccompanied by disturbing motor movements One hour following the initiation of the nembutal narcosis two small openings were made by trephine in the right frontal region of the skull 0 5 cm distant from the sagittal suture and on each side of the coronal suture 1 5 cm apart the dura was then cut at these points The electrodes were of lucite $\frac{1}{4}$ inch in diameter and threaded to facilitate insertion A single wire passed through the center terminating in a ball of solder 2 millimeters in diameter The electrodes were screwed into place bringing the ball of solder in direct contact with the surface of the cortex Duco cement was applied around the insertion of the electrodes and the wound was closed with clips The recording instrument used was a *Grass* 2 channel ink writer The amplifiers were calibrated at 100 microvolts and the gain controls set at convenient level Records were made intermittently at speeds of 1 5 3 and in some cases 6 centimeters per second At the beginning and at the end of each experiment the amplifiers were calibrated

Since the nembutal anesthesia itself may cause some alteration of brain waves (15 16, 17, 18 19) a number of electrocorticograms of the narcotized untreated animals were taken This action is shown in Fig 1 in which during 116 minutes following intraperitoneal injection of 30 mg per kg the pattern of the brain waves remains almost unchanged in amplitude and frequency This picture may serve as control for the electro corticograms obtained after atabrine

Atabrine in a 2% aqueous solution was administered either intraperitoneally or intravenously The 2% solution had a pH of 5 6 Control tests using equal quantities of mono sodium phosphate, which showed an even stronger acid reaction of 4 5 pH had no effect upon the electro encephalogram The administered quantity amounted to 10-20 mgm of atabrine per animal so that approximately 6 12 mgm per kgm was given The intravenous injections were made very slowly since the fatal effect of atabrine is largely dependent upon the speed of the injection (7 8) In our tests on cats under nembutal narcosis we never observed convulsions and the animals survived without any ill effects the above intravenously administered atabrine doses

Atabrine determinations in tissues were made according to the single extraction method of Brodie and Judenfreund (20)

RESULTS *I Frogs* Electro encephalographic observations on thirty frogs demonstrated (see Fig 2) that following the injection of 0 5 mgm of atabrine dihydrochloride into the thoracic lymph sac the amplitude and the frequency of the electric waves diminished This change affected both the hemispheric surfaces as well as the optic lobes An additional injection of 0 5 mgm given 15 minutes later produced a further reduction in the electric potential which lasted for about an hour One or 2 mgm injected in a single dose altered the electrical activity of the frog brain in a similar manner for more than one hour

II Cats The effect of atabrine upon the electro corticogram usually became manifest about two hours following the intraperitoneal or intravenous injection but occasionally occurred earlier, especially following the intravenous injection

It consisted in a suppression of the fast waves (10–11 per second) characteristic of light nembutal anesthesia and lasting usually for 1 to 2 hours or longer, showing the slow, low amplitude waves (6–7 per second) in a similar manner as in the recordings obtained on frogs (Fig. 3 and 4). In some of the experiments the original activity was restored by the third hour; following larger doses, however, (12 mgm. per kgm.), the effect of atabrine was observable up to 4 hours later.
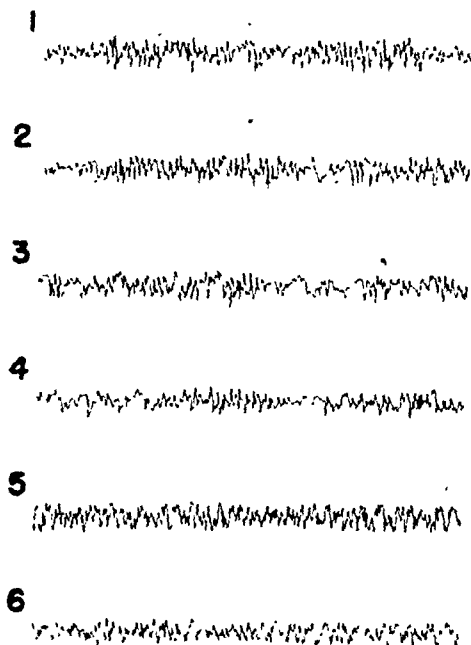
FIG. 1. ELECTRO-CORTICOGRAM OF A CAT IN NEMBUTAL-NARCOSIS DURING FOUR AND ONE-HALF HOURS

Cat 1.7 kgm., 30 mgm./kgm. nembutal intraperitoneally Record (1) taken *30 minutes*; Record (2) *79 minutes*; Record (3) *106 minutes*; Record (4) *138 minutes*; Record (5) *190 minutes*; Record (6) *216 minutes* after intraperitoneal nembutal-injection. All records show substantially the same average amplitude, frequency and shapes. Ampl. 7 mm. Cal. 100 micro-volts, speed 1.5 cm. per 1 second.

Special attention has been given to the question of the distribution of atabrine in the blood and in the brain in order to determine to what extent the concentration of atabrine in blood or brain may be responsible for alterations in the electro-corticogram. Atabrine disappears quickly from the blood while it can still be demonstrated in the organs, especially in the liver and in the brain for longer periods (6, 9). It appears therefore that the electro-corticographic changes depend upon the atabrine content of the brain and not on its concentration in the blood. This is to be assumed since the altered electro-corticogram

appears as a rule about 1 to 1½ hours following the intravenous injection when the atabrine blood content is already considerably reduced (Table 1)

Further investigation into the question of atabrine content and distribution in the brain agree that relatively little of the dye is deposited in the brain as compared to other organs, but like other organs it can retain the substance for some time. While *Hecht* (4) found atabrine in the brain and particularly in the cortex only following fatal doses and the cerebrospinal fluid always free of ata
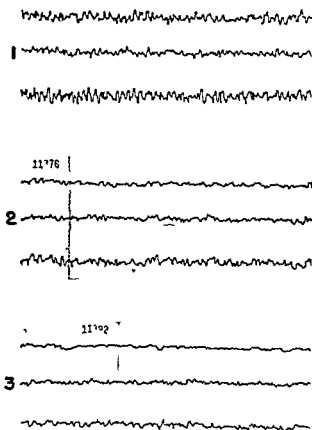
FIG 2 EFFECT OF TWO INJECTIONS OF 0 5 MG ATABRINE INTO THE THORACAL LYMPH SAC OF A PITHED FROG OF 35 GRAM

Record (1) *before* atabrine injection, Record (2) taken *5 min after first* injection of
0 5 ... after
f the
left
s fol-
icro-

volts, speed 3 cm per 1 second

brine, our investigations show in corroboration of analyses by *Dearborn, Kelsey, Oldham and Geiling* (9) that the brain can retain considerable quantities of atabrine, namely 3 3 to 6 microgram per gram, following small nontoxic doses of 5 to 10 mgm per kgm

DISCUSSION   A definite depressive effect of atabrine upon the electrical brain activity was observed in approximately 66% of our cat experiments and doubtful effects in the remaining one-third of the test animals.   On the basis of the large

number of conclusive tests observed, we believe that we can conclude that atabrine even in non-toxic doses can exercise a depressive effect upon the cortex and appears also to increase the effect of nembutal on the corticogram. It is well known that anoxia produces some alterations in the brain potentials (13, 14). Since atabrine in fatal doses impairs the respiratory center the possibility should be considered that the observed changes in the brain waves following
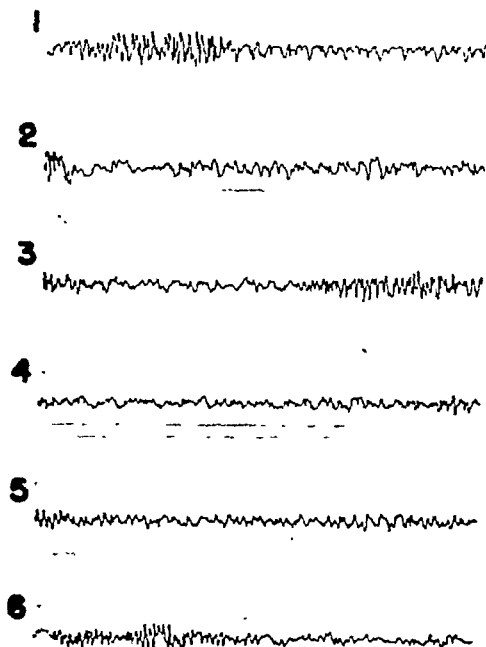
FIG. 3. EFFECT OF INTRAVENOUS INJECTION OF 20 MGM. ATABRINE ON THE CAT ELECTRO-CORTICOGRAM

Cat 1.75 kg., 30 mgm. per kgm. nembutal intraperitoneally. One hour later 20 mgm. atabrine dihydrochloride, in 2% solution intravenously. Record (1) *before* atabrine injection. Record (2) 1 minute, Record (3) 17 minutes, Record (4) 31 minutes, Record (5) 74 minutes, Record (6) 149 minutes after atabrine injection. Record (3) and especially (4) show definite alteration of waves; in record (4) the waves of higher amplitude disappeared entirely, (5) and (6) show beginning recovery. Ampl. 8 mm. 100 micro-volts, speed 1.5 cm. per 1 sec.

atabrine application might be due, at least partly, to anoxia. In our experiments, however, only doses of atabrine were used which did not visibly affect the respiration. This is in agreement with *Unna* (8), who showed that the intravenous injection of sublethal doses did not influence the respiration of anesthetized cats.

The reduction in the electrical activity of the brain following administration of atabrine agrees with other findings which point to a depressing action of this drug

upon certain brain functions Thus, *Hecht* (4) demonstrated in cats in which
a fever was produced by means of B coli vaccine, that atabrine in doses of 0 1
gram per kgm reduces the body temperature by 0 6 to 0 9 degrees and that the
maximum effect occurs about two hours following administration, *Chopra*(2)
reported that the reduction of temperature ranged up to 1°C in rabbits with
induced fever lasting for about twelve hours *Unna* (8) recently found that the
sudden drop in blood pressure and the cessation of the respiration following
fatal doses of atabrine is apparently due to paralysis of the vasomotor and
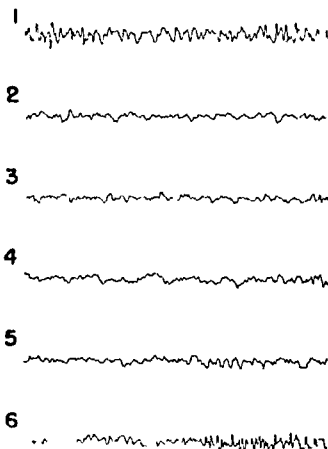
FIG 4 THE SAME EXPERIMENT AS IN FIG 3 WITH GREATER SPEED (3 CM PER 1 SECOND)
Record (1) *before* atabrine injection Record (2) 3 minutes Record (3) 23 minutes Record
(4) 51 minutes Record (5) 108 minutes Record (6) 158 minutes after atabrine injection
A very distinct alteration of brain waves in altitude and frequency in records 2 3 and 4,
in 5 and 6 beginning recovery

respiratory centers and may be counteracted by picrotoxin The effect of ata
brine in producing central depression appears along with blocking of electrical
brain potentials, to precede the reduced irritability and the subsequent motor
paralysis, in frogs

SUMMARY

1 Atabrine in doses of 5, 10, 12 mgm per kg given intraperitoneally or
intravenously can alter the electro corticogram of cats in nembutal anesthesia

The rapid wave frequency usually disappeared and only slow waves of low amplitude remained.

2. This central depressant action of atabrine seems to be independent of the atabrine blood concentration and appears to be related to the atabrine content of the brain. This effect was observed within a few minutes following intravenous injection, but usually did not appear until one hour or longer after the injection; it persisted, depending upon the dose given for 1–4 hours.

TABLE 1

*Distribution of atabrine in blood and tissues**

BLOOD SAMPLES	TIME AFTER I.V. INJECTION	γ/cc.	TISSUES	TIME	γ/GM.

Experiment No. 1: A 2.2 kg. cat was given 20 mg. atabrine in 1 cc. intravenously and encephalo-corticograms were recorded

	min.			min.	
	Control	0	Liver	140	60
	15	2.5	Brain	140	6
	35	2.2			
	60	1.5			
	120	0.8			
	140	0.6			

The boldface figures belong to abnormal electro-corticogram; this was still abnormal after 140 minutes when the cat was sacrificed by bleeding

Experiment No. 2: A 2.9 kg. cat was given 50 mg. atabrine in 2.5 cc. intraperitoneally and encephalograms were recorded. Blood and tissue atabrine levels were as follows

	TIME AFTER I.P. INJECTION				
	10	2.0	Liver	260	77
	25	2.0	Brain	260	3.3
	45	2.0			
	140	1.8			
	205	1.5			
	235	1.0			

The encephalogram was abnormal within 30 minutes, with maximal effect at about 1 to 2 hours (boldface figures); at termination of the experiment the effect was clearly decreasing

* We are indebted to Dr. R. H. Silber for these analyses.

3. A similar atabrine action was seen in the electrocorticogram of pithed frogs following the injection of 0.5 mg.–1 mg. atabrine. 2 mgm.–3 mgm. of atabrine injected into the thoracic lymph sac blocked the righting reflexes and reduced the motility of the animals; it was followed by a reversible paresis lasting several hours or by irreversible paralysis in spite of a continued heart action, finally leading to death.

4. Our results tend to support clinical observations of central disturbances occurring during, or following the administration of atabrine.

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THE INFLUENCE OF THIAMIN-DEFICIENCY ON WORK PERFORMANCE IN RATS

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The influence of specific nutritional deficiencies on the ability to perform hard physical work is a topic of much interest particularly at the present time, when greatly increased demands are being made on the physical endurance and fitness of large sections of the population. A number of studies in man have established beyond doubt that under certain conditions a deficiency in vitamins of the B-complex may result in an impairment of physical fitness (1, 2). However, no systematic investigation in experimental animals has been reported, due probably to the technical difficulties of measuring muscular endurance in non-anesthetized intact animals, particularly smaller species such as the rat, which however are the animals of choice for nutritional studies. Nevertheless, such a study would seem highly desirable on account of the limitations imposed on investigations of experimental vitamin-deficiencies in man. It is well known, that as a rule vitamins exhibit a pharmacodynamic effect only in a deficiency state (3), and the work of Keys and Herschel (4), in man has proved that this general principle holds also true for their effect on muscular fatigue. It is, on the other hand, almost impossible to produce and maintain in man, experimentally, vitamin deficiencies for any length of time, not only because of the possibility of complications due to unexpectedly severe reactions, but also on account of the difficulty of feeding a generally unpalatable and monotonous diet over a prolonged period of time. We need only consider the fact, that the clinical syndrome of pyridoxin and pantothenic acid deficiency has not yet been established in man, although both deficiencies have been extensively studied in laboratory animals and both vitamins have been freely available for a number of years. For these reasons it seems advisable to study the influence of certain vitamins or other dietary factors on fatigue first in deficient animals before undertaking the laborious task of a rigidly controlled study in man.

The study of muscular endurance in animals is technically not easy. While a human volunteer will perform an imposed strenuous exercise until completely exhausted and will thus establish a reasonably accurate end-point, this is not the case in the experimental animal, which will exercise to the limit of its capacity only if forced to do so.

The widely used method of a treadmill forces the animal to perform to the

utmost of his ability, however, the form of stimulation applied in this procedure bears no relation to the amount of energy expended by the animal While it is possible to vary the amount of exercise by adjusting the speed and the incline of the treadmill, this is practical only in man and, possibly, in the dog, but not in the rat Furthermore, frequent running on the rough tracks of a treadmill tends to injure the feet of an animal which is struggling against this forced type of exercise and the minute cuts and bruises thus produced may lead to a second ary anemia through repeated, though apparently negligible losses of blood, this is likely to complicate the interpretation of the results

A method of exercising which overcomes most of these objections and which is exceptionally well suited to the use in small animals is that of swimming with an attached weight This method, first employed by Klejn and Van Wyndgarden (5) in their studies of adrenalectomized rats, has the advantage that it completely eliminates the possibility of a mechanical injury, furthermore, the weight, which the swimming animals is made to carry, provides a simple way of varying and measuring the amount of work performed

The usual difficulty of determining the endpoint in this type of experiments appears on first impression to be minimized with this method since one might assume that the animal will make every attempt to keep himself above water until completely exhausted, the moment when he can no longer swim and sinks below the surface should thus mark the endpoint Unfortunately, however, this assumption is not borne out by the facts The majority of the rats soon develop a tendency to insert short rest periods between stretches of swimming If they are not made to carry a weight, they accomplish this by permitting them selves to float with a minimum of motions Any prolonged sinking below the surface under such circumstances could properly be regarded as a sign of com‑ plete exhaustion, however a test conducted in this manner would most likely extend over many hours since the rat possesses remarkable endurance and will keep on swimming for many hours, it would thus become almost impossible to conduct an investigation on a group of animals sufficiently large for statistical evaluations It is therefore necessary to attach to the animal a weight which is heavy enough to make it impossible for him to remain on the surface without active swimming movements However, if the animal is made to carry such an extra weight, he soon learns to take a rest by permitting himself to sink to the bottom of the tank, even if in doing so he occasionally traps himself below the surface and may drown long before having reached a state of complete muscular exhaustion

The determination of the critical endpoint offers for the above reasons almost as many difficulties with the swimming method as with other methods of forced exercise To overcome these difficulties, an apparatus was devised which not only permits a continuous change of the weight carried by the animal while he is swimming but adjusts it automatically to his maximum carrying capacity With such a device the animal learns after a few experiments that even a heavy load will force him to exercise only as long as he can but will not drown him as his powers weaken Consequently, his swimming movements change soon from a

frantic struggle for life to a regular and coordinated motion.   The weight, carried at any given moment is an indication of the animal's strength and serves as a reasonably objective measure of its physical condition.

A sketch of the equipment is given in figure 1. D_1–D_2 is a beam balance supported on 2 knife edges E–E.   The rat is suspended at one end of the balance arm (D_2) by means of a wire-cable I and an elastic harness J.   A weight, slightly in excess of the maximum load which the animal is to carry, is attached to the harness and the entire unit is fastened to the chest of the animal.   All weight in excess of what the rat is capable of supporting is counterbalanced by a variable length of chain X, attached to the opposite end of the balance arm (D_1).   The free end of the chain is wound around the drum O, which is driven by a reversible
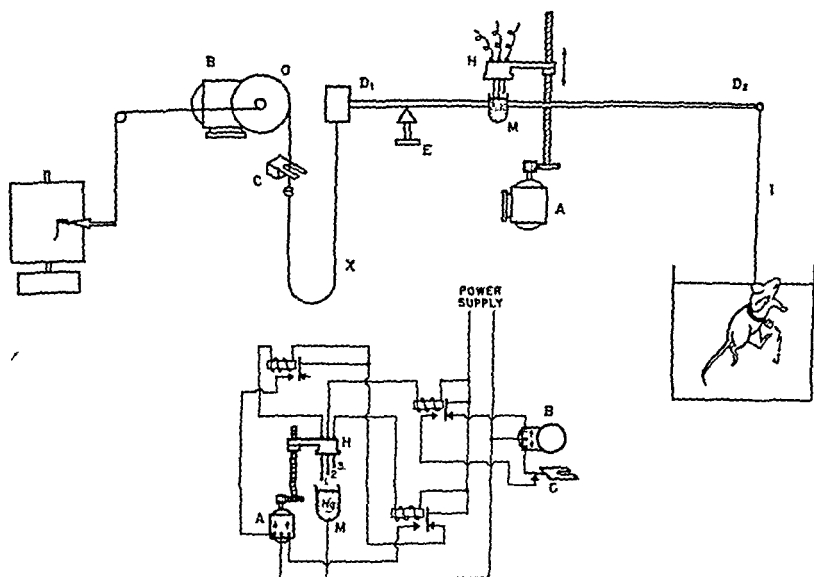
FIG. 1. SCHEMATIC DIAGRAM OF SWIMMING APPARATUS

motor B.   The movements of the motor B are controlled by the position of the swimming animal: If he sinks, the weight on D_1 is increased and the load on the animal reduced; if, on the other hand, he swims vigorously and maintains his position with respect to the surface, the weight on D_1 is decreased and the load which the animal has to carry is correspondingly increased.   A cutoff switch C stops the chain at a point at which the load imposed on the animal reaches a predetermined maximum.

The operation of motor B is controlled by 3 platinum contacts H_1, H_2, H_3. These are moved up or down by an auxiliary motor A to make contact in a cup of mercury M fastened to the beam balance.   Contact H_2 controls the loading motor B, decreasing the chain length on D_1 when open and increasing it when

closed   The swimming performance of the rat governs the position of contact
H.   Even if the rat makes a permanent change in its swimming position with
respect to the surface of the water, the contact motor A maintains through
lowering or raising of the entire group of contacts H_1, H_2, H_3 the same relation
of contact H_2 to the mercury   Contact H_3 controls auxiliary motor A, moving
the points up when contact is made and down when contact is broken   If H_1
contact is broken, the auxiliary motor A is stopped completely   This serves as
a safety measure   Its purpose is to stop the downward motion of the contact
motor when the rat drifts persistently towards the bottom and remains there
for a long period, as is the case when he gets tired   If under such conditions the
contact motor would not be stopped, the distance between contact H_2 and the
mercury would be reduced to a degree, where a slight upward movement of the
rat would cause motor B to increase the load on the animal although it was still
under water   The speed of the adjustment is relatively slow, so that a transient
change in the position of the animal, such as when he drifts to the bottom, or
lifts himself with a few energetic strokes has no appreciable influence on the
position of the contacts

EXPERIMENTAL   To measure the performance of a rat, the following procedure is fol
lowed the hair is removed from the entire body, since the presence of air bubbles in the fur
tends to buoy the animal and introduces an additional variable, when experiments are
extended over several weeks, the clipping is repeated whenever necessary   As a further
protection against air bubbles the water is poured into the swimming tanks at least 12 hours
before the test and is kept during this time at 30°C , this temperature is also maintained
during the test   The swimming tanks are made of glass and are 18″ high and 10″
in diameter

The clipped rat is weighed to ± 0.5 gm , put into an elastic, weighted harness, placed into
the tank and permitted to swim   Our device permits conducting the test in two ways,
offering different types of exercise   One, named in the following ' endurance test ' consists
of exercising the rat with the heaviest load which he can comfortably carry and to adjust
this load subsequently to his waning strength   An experiment conducted in this way may
extend over several hours, until the animal becomes exhausted   However, we have usually
terminated the test after 15–30 minutes since it was observed that the most significant
changes occur during this period   Indeed, the time between the start of the experiment
and the point when the swimming record drops 1 cm below the starting level, provides a
reasonably accurate estimate of the performance of the animal which may be expected
during the following hours   The results of the endurance test are expressed in gram min
utes obtained by planimetric measurement of the area under the swimming curve, or in
' time of optimal performance ' , represented by the time during which the animal maintains
its swimming curve within 1 cm of the starting level   Both values parallel each other
closely

The alternative to the "endurance test is the "peak performance' test   In this the
animal is started with a very light load, which is steadily increased until within 1–2 minutes
the maximum tolerated load has been reached and the animal sinks under the surface, at this
moment the test is immediately terminated   The results are expressed in the maximal
weight which the animal was able to carry

The results of the "endurance ' and the ' peak performance ' tests show usually the same
trend but each test examines somewhat different stamina of the animal, the "endurance"
test may be compared to a marathon race, while the ' peak performance ' test resembles a
100 yard run   A variation in muscular performance may show up better in one or the other
modification and for this reason both should be tried until the more sensitive one has been

After having investigated the various factors influencing swimming tests in normal animals we proceeded to study the effect of thiamin deficiency on muscular performance.

THIAMIN-EXPERIMENTS. The influence of thiamin deficiency on the muscular performance was studied in a total of 120 rats, which were sub-divided in groups of 12–16 animals, one-half of which served as pair-fed controls. The rats varied in weight from 30 gms. to 400 gms. with the greater part in a weight-range of from 210 to 290 gm. This age group represented a compromise between the optimum for deficiency studies and that for swimming experiments. The younger rats developed more easily and rapidly the vitamin deficiency while the adult ones were preferable for the exercise tests. There was, however, no qualitative difference between these widely varying age groups and the findings reported in the following are typical of the entire investigation.

The procedure followed in these experiments was as follows: a group of 12–16 rats, matched for weight and sex, was subjected to a few preliminary swimming tests, in order to eliminate unsuitable animals. After the final group had been formed, all animals were standardized for their swimming performance and the test group was then placed on a diet, which was complete in every respect except for thiamin.[2] Food consumption and body weight were determined daily and the food intake of the control-group was restricted to that of the deficiency group. Swimming tests were performed with both groups, in the early part of the experiment every second day and in the later part, when the body weight of the deficient animals had begun to decline and deficiency signs had developed, at daily intervals. The time necessary to produce thiamin deficiency varied widely, depending on the age of the rats; some of the older animals failed completely to develop the typical convulsions and manifested the progressing deficiency chiefly in a loss of weight; however, even in these atypical thiamin deficiencies an impairment of muscular performance could be observed.

After an animal showed signs of deficiency, it was treated with thiamin hydrochloride, which was injected intraperitoneally or intramuscularly in massive doses, usually 250 γ per rat per day; the pair-fed controls were given an identical course of treatment. The length of time during which the animals remained on the deficiency diet varied greatly, as did the severity of the deficiency at the time of initiation of treatment. Since we were interested in studying different stages of the disease, early as well as fully developed deficiencies were examined. However, rats which had progressed to the convulsive stage could for technical reasons not be subjected to swimming tests; in these instances one day had to be skipped and the test had to be postponed until several hours after injection of the first dose of thiamin.

Thiamin-treatment with massive dose was given for 2–6 days; during this time the deficient as well as the control rats were restricted to the amount of food which they had consumed on the day immediately preceding the termination of the deficiency. This was done to differentiate between possible action of the vitamin and the combined effect of vitamin administration plus a sudden great

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[2] The composition of the diet was as follows: Casein, vitamin free, 18%; Corn starch, 53%; Autoclaved brewers' yeast, 15%; Salt mixture USP No. 1, 4%; Butter fat, 8%; Cod liver oil, 2%.

increase in food intake.   After a few days of this restricted regime the animals
were permitted free access to food and the thiamin deficient diet was replaced
with a complete stock diet.

The conditions in these experiments varied so widely, that it is impossible to
present all individual experiments in tabulated form; the following graphs of one
individual rat and of one group of rats may serve as typical examples.   Figure 4
demonstrates the effect of thiamin deficiency in a male rat, which weighed at the
beginning of the experiment 208 gm. and regained this weight at the end of the

FIG 4 EFFECT OF THIAMIN DEFICIENCY ON WORK PERFORMANCE

ency and during the period of recovery.

experimental period of 7 weeks, after it had temporarily dropped to 180 gm. at
the height of the deficiency.   The first signs of thiamin deficiency developed after
23 days.   At this time, its swimming performance deteriorated rapidly, until it
reached a low 7 days after the first decline in weight had been noted.   At this
point the treatment with 250 γ thiamin hydrochloride was begun and continued
for 6 days, with food restricted during this period to 4 gms. daily.   It can be seen
from the graph, that the swimming performance increased almost immediately.
The "optimal swimming performance" reached almost the original level, while

in the more severe "endurance-test" about one half of the original performance was regained. After the rat had been given free access to food, the original level was reached in both tests.

Figure 5 shows the influence of thiamin deficiency on a group of 4 rats and a pair-fed control group. The initial average weight was 250 gm. In order to obtain a better comparison, the changes in the swimming performance of each individual animal were expressed in percentage deviations from the starting value; averages of all such figures were then calculated and plotted on the graph. It can be seen that the thiamin deficient group decreased in swimming per-
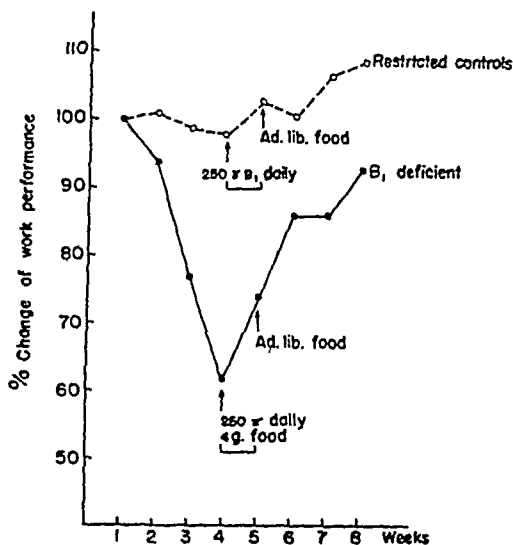
FIG. 5. AVERAGE WORK PERFORMANCE OF A GROUP OF FOUR THIAMIN DEFICIENT AND FOUR PAIR-FED CONTROL RATS

Abscissa, time in weeks; Ordinate, average variation in work performance as expressed in per cent deviation from the normal performance of each individual rat. The average weight of the rats at the beginning of the test was 250 grams. Heavy line, work performance of thiamin deficient group; Dotted line, work performance of pair-fed control group.

formance from 100 to 62%; during the same time the pair-fed controls decreased only from 100 to 98%; upon intraperitoneal administration of thiamin to both groups (250 γ daily for 6 days), the performance of the deficient group rose 12%, the pair-fed control group 5%; after both groups were given free access to food, the thiamin-deficient group increased its performance an additional 19%, the control group 6%.

The following is a summary of all experiments: a total of 120 rats were used, of which 69 were placed on a thiamin deficient diet, while 51 served as pair-fed controls. Of the 69 deficient rats, only 4 failed to show a marked impairment of swimming performance, as the deficiency progressed; 16 rats died either accidentally or from the deficiency, before thiamin administration was started. Of

the remaining 49 rats, which permitted completion of the entire experiment, 43 responded to thiamin administration with a definite improvement of swimming performance while 6 rats failed to respond

Discussion From the above as well as similar experiments we conclude that 1) thiamin deficiency results in a rapidly progressing impairment of work performance, 2) this deterioration of physical fitness is not caused by inanition due to decreased food intake, 3) parenteral or oral administration of large doses of thiamin, even without increasing the food intake, improves markedly the work performance of the deficient animals, 4) thiamin administration fails to have such an effect on non-deficient rats Substitution of the deficient diet with a normal diet without additional injection of large doses of thiamin causes a similar restoration of the impaired work performance, however, the effect is less dramatic and requires several days, whereas the parenteral administration of very large doses of thiamin and simultaneous free access to food restores the impaired performance within 1–2 days, several days before the body weight has returned to its original level

Our experiments are in complete agreement with the observations in man reported by (1, 2, 3) We have been able to show that during the course of a thiamin deficiency the physical fitness of rats deteriorates remarkably fast and reaches the lowest level only a few days after the body weight of the animals has started to decline Normal animals, on the other hand, are not benefited by the administration of even excessive doses of thiamin A considerable reduction of food intake appears to be without marked influence on the work performance, as long as it does not result in the development of thiamin deficiency

The experiments reported above are further evidence for the importance of an adequate thiamin intake for optimal work performance The question, whether other nutritional deficiencies are likely to cause a similar impairment of physical fitness or whether this is specific for thiamin deficiency, is still open While the pair fed controls in our experiments show that a simple reduction in the caloric intake of an otherwise adequate diet is not likely to exert a detrimental influence on work performance, we are inclined to believe that a deficiency of at least some of the other vitamins of the B complex will manifest itself in a similar manner as a thiamin deficiency Experiments along these lines are under way and will be reported later

SUMMARY

1 A method for the recording of the work performance of swimming rats is described This method permits the discovery of minor impairments in the physical fitness of these animals and affords a means to measure and record quantitively the changes of muscular performance

2 Various factors influencing these tests have been investigated and their importance is analyzed

3 Administration of a thiamin deficient diet results in a rapid and marked decrease of physical fitness

4 Administration of thiamin restores promptly the impairment of work-performance due to thiamin deficiency

5. This reduction of work-performance is not caused by a reduced intake of food.

6. Administration of thiamin, even in large doses, to non-deficient animals is without beneficial effect on their work performance.

7. Reduction of food intake alone is not accompanied by a decrease in work-performance, as long as a nutritionally adequate diet is provided.

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BIOLOGICAL ASSAY OF POSTERIOR PITUITARY

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The purpose of this paper is to present a comparatively simple method by which the oxytocic activity of posterior pituitary solutions can be determined with hitherto unattainable precision. The widely recognized disadvantages of the guinea pig uterine method have been set forth by Morrell, Allmark and Bachinski (1). They devised a new method based on the quantal response of eight uterine strips and designed so that the reliability of individual assays could be determined. In their assays of solutions of 'unknown' potency they obtained errors up to 35 per cent with an average error of 5.98 ± 7.13 per cent.

The chicken blood pressure test for oxytocic activity reported by Coon (2) and studied by Smith (3) is simpler and more economical. This test is carried out by injecting intravenously varying doses of standard and unknown until doses are found which produce equal, submaximal falls in blood pressure. Exactly equal falls in pressure are rarely obtained and even when they are they may be misleading because of chance variations in response or because of changing sensitivity to the drug as the experiment progresses. It is difficult to determine the experimental error of each assay. The proper interpretation of the data depends largely on the judgment and experience of the assayist. Coon (2) claims only   as great a degree of accuracy as can be expected in biological assay.' This gives little information of the precision to be expected.

Smith and Vos (4) devised a more satisfactory procedure for conducting the chicken blood pressure test in that the results and experimental errors of individual assays are computed mathematically. Their procedure involves the randomized administration of each of four doses four times, making a total of sixteen injections with a ten minute interval between doses. Their method is designed according to the precepts of Bliss and Marks (5, 6) and is similar to that employed by Schild (7) for his assays of histamine on isolated guinea pig gut. Smith and Vos (4) obtained errors up to 18.2 per cent with an average error of 6.9 per cent in their assays of posterior pituitary solutions of 'unknown' potency.

In biological assays where successive doses are given to the same test object the chief difficulty is the elimination of the effect of changing sensitivity from the experimental comparison. Schild's (7) or Smith and Vos' (4) method accomplishes this to a degree since the four doses are repeated at different sensitivity levels. However any change in sensitivity which occurs during the period required for the administration of four doses obviously causes variation in responses which would contribute to the error of the assay. This change is often great in the chicken blood pressure test for oxytocic activity of posterior pituitary

and probably accounts largely for the magnitude of the errors obtained by Smith and Vos (4).

Vos (8) reported a method for the assay of ergonovine on rabbit uterus in which he utilized the latent period as the response. His experimental design and method of interpretation, theoretically at least, eliminated almost entirely the effect of changing sensitivity from the experimental comparison. That he was quite successful is evidenced by the precision of his assays of ergonovine solutions of "unknown" potency. He obtained a maximum error of 9 per cent with an average error of 3.8 per cent.

The present method involves the application of the experimental design employed by Vos (8) for the assay of ergonovine on rabbit uterus to the chicken blood pressure test for oxytocic activity of posterior pituitary extracts reported by Coon (2).

EXPERIMENTAL. For recording blood pressure a Harvard membrane manometer[1] was used in place of the mercury manometer employed by Coon (2). This recording device eliminates the inertia of the mercury column thereby influencing to a lesser extent the evanescent fall in blood pressure produced by posterior pituitary. The flow of liquid back and forth in the cannula as blood pressure changes is also minimized. This aids in preventing clots and reduces the amount of citrate that enters the blood stream. The manometer was adjusted so that 1 mm. excursion of the writing point represented a change in pressure of approximately 2 mm. of mercury. The actual magnification of the response is unimportant just so it is enough to allow accurate measurement of the record produced and the same magnification is used throughout the assay. Further magnification of the response has no more effect than multiplying all responses constituting an assay by a common factor. By means of an adjustable screw clamp, placed on the rubber tube connecting the cannula with the manometer, the excursions of the writing point produced by the heartbeat were reduced to an amplitude of 1 to 2 Cm. before beginning an assay.

8.5 per cent sodium citrate solution was used as anti-coagulant in place of the 5 per cent used by Coon (2). This appeared to further reduce the incidence of clotting.

A branch of the brachial vein (underside of wing) was utilized for intravenous injections instead of the crural vein used by Coon (2). This is somewhat more convenient to the operator. There is also less chance of jostling the arterial cannula when doses are administered. The injections were always made at the junction of two branches of the vein so that the blood flowing from one branch helped to wash the drug into the circulation.

White leghorn roosters varying in weight from 1.1 Kg. to 1.8 Kg. were employed for this study. During the course of the experiments 18 roosters were prepared for assay of posterior pituitary solutions. Two of these were discarded as unsuitable because of very rapid development of tachyphylaxis.

The assay procedure evolved and used in the present experiments is as follows. A white leghorn rooster is selected, anesthetized, set up for intravenous injection and the blood pressure recorded as directed by Coon (2) except that, as indicated above, a Harvard membrane manometer is used in place of a mercury manometer, 8.5 per cent sodium citrate is used as anticoagulant instead of 5 per cent and a branch of the wing vein (brachial) is utilized for injections instead of the crural vein. One cc. of U.S.P. standard posterior pituitary solution is diluted to 10 cc. with 0.9 per cent NaCl solution and the solution to be tested is diluted with the same diluent to an expected potency of 100 per cent of the standard. Stronger solutions are used when the required dose exceeds 0.4 cc. A dose of the standard is found by preliminary trial which will produce a submaximal response equivalent to a fall in blood pressure of about 30 to 50 mm. of mercury. This standard dose is

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kept constant and is administered alternately with doses of the unknown which are varied so that some give responses less than the standard, others give responses approximately equivalent to the standard and others give responses greater than the standard   Actually the dose of the standard may be increased during the course of the assay if the rapid development of tachyphylaxis makes it necessary in order to maintain recorded responses above 10 or 15 mm   The unknown doses must then be proportionately increased and each section of an assay involving a certain dose of the standard must begin and end with that dose   Likewise, in the event of a clot the cannula may be washed and the assay continued just so each unknown dose used in computing the result is preceded and followed by a constant dose of standard   This versatility of the experimental design enabling one to deal with interruptions in the assay without materially affecting the result, was not pointed out by Vos (8)

   The dose of the unknown which produces a larger response than the standard should be no more than twice as large as the dose which produces a response less than the standard   The intermediate dose may be varied if desired to try to produce responses exactly equivalent to those of the standard, thereby producing graphic evidence of the strength of the unknown according to the principles of matching responses

   The assay must be run on a definite time schedule   The interval between doses in the present assays was three minutes in all cases   Coon (2) recommends an interval of 3 to 5 minutes in his procedure while Smith and Vos (4) used a ten minute interval   The shorter interval makes possible the administration of a greater number of doses in a given time thereby increasing the accuracy of the result   An interval longer than three minutes may be used if at any time it is required to allow the blood pressure to return to normal

   To summarize, the doses are administered at three minute intervals in the following order S U$_1$ S U$_2$ S U$_3$ S U$_1$ S U$_2$ S U$_3$ S etc where S represents the constant dose of standard and U$_1$, U$_2$ and U$_3$ represent the low, medium and high doses of unknown respectively   The number of times the doses are repeated is governed by the accuracy desired

   A protocol of an assay involving 15 doses of unknown and 17 of standard is shown in table 1

   It was necessary in this assay to increase the doses to stay in the proper response range   The doses were increased starting with dose number 49   If clotting occurs at any time the clot may be washed from the cannula and the assay continued as before   Any data produced by a dose of unknown not preceded and followed by a constant dose of standard without interruption, must be eliminated from the computation of the result   By the use of the membrane manometer and 8 5% sodium citrate as anticoagulant clotting hardly ever occurs during an assay.   If several assays are run on one rooster the cannula should be washed between assays thereby further reducing the likelihood of clotting

   RESULTS   Calculations   The assay data are interpreted precisely as indicated by Vos (8) for his ergonovine assays on rabbit uterus except that in the present assays the responses are not expressed as logarithms [2]   Smith and Vos (4) have shown that the depressor response of chicken blood pressure increases linearly

   [2] The results of the present assays were also computed after expressing the responses as logarithms   Only insignificant differences were obtained as would be expected from the experimental design   The use of mm response in the calculations for eight assays resulted in an average per cent error of 2 71 while the use of log response gave an average per cent error of 2 45   Average per cent standard errors were 3 03 and 2 90 respectively

with the logarithm of the dose of posterior pituitary. Tables 2 and 3 show the details of the calculations as applied to the data in table 1.

In table 2, columns 2 and 3, the logarithms are of the doses times 10 to avoid negative logarithms. In columns 4 and 6, table 2, the original recorded responses from table 1 have been multiplied by 2 to avoid the introduction of decimals when the standard responses are interpolated. If the recorded responses were measured to the nearest 0.5 mm. multiplication by four would accomplish the same result. This only simplifies the arithmetic and does not affect the final result.

TABLE 1

Protocol of assay

9-23-43.   Rooster 1.4 Kg.   200 mgm./kgm. Na Phenobarbital I.M.

DOSE NUMBER	PREPARA- TION	DOSE	RECORDED RESPONSE	DOSE NUMBER	PREPARA- TION	DOSE	RECORDED RESPONSE
		cc.	mm.			cc.	mm.
32*	S	0.20	30	48	S	0.20	17
33	U	0.20	36	49	S	0.30	33
34	S	0.20	30	50	U	0.36	36
35	U	0.15	23	51	S	0.30	27
36	S	0.20	28	52	U	0.30	27
37	U	0.17	24	53	S	0.30	23
38	S	0.20	27	54	U	0.30	15
39	U	0.20	29	55	S	0.30	22
40	S	0.20	26	56	U	0.28	26
41	U	0.25	32	57	S	0.30	25
42	S	0.20	24	58	U	0.36	29
43	U	0.20	25	59	S	0.30	25
44	S	0.20	22	60	U	0.26	25
45	U	0.15	17	61	S	0.30	22
46	S	0.20	20	62	U	0.20	19
47	U	0.20	22	63	S	0.30	22

* A previous assay involved the first 31 doses administered to this rooster.

The estimated potency and the standard error for the assay illustrated, 112.6 ± 2.13%, is in satisfactory agreement with the true potency of 110 per cent to which this unknown had been made by dilution of the standard solution.

EXPERIMENTAL RESULTS. In order to determine the precision obtainable by this method and to study the validity of the standard errors 8 solutions of posterior pituitary of known potency were assayed. To eliminate personal bias the true potencies of the unknowns were not made known to the assayist until after the results were computed. An experiment on each of the eight solutions involved the administration of 12 to 17 doses unknown. By designating each half of an experiment as a separate and complete assay results of two assays on each solution were obtained where only a relatively small number of doses were utilized for each. The results of two such assays on each solution are shown in table 4. This table shows the precision to be expected when 6 to 9 doses of

unknown are employed in an assay. The maximum per cent error is 8 0 while the average is 3.2. By designating each experiment as a single assay the single value obtained for each solution resulted from the use of twice as many doses.

TABLE 2

Analysis of data in table 1

DOSE NUMBER	LOG DOSE × 10		2 × MM RECORDED RESPONSE			X $(X_s - X_u)$	Y $(Y_s - Y_u)$
			Standard		Unknown Y_u		
	Standard X_s	Unknown X_u	Observed	Interpolated Y_g			
32	0 301		60				
33		0 301		60	72	0	−12
34	0 301		60				
35		0 176		58	46	0 125	12
36	0 301		56				
37		0 230		55	48	0 071	7
38	0 301		54				
39		0 301		53	58	0	−5
40	0 301		52				
41		0 398		50	64	−0 097	−14
42	0 301		48				
43		0 301		46	50	0	−4
44	0 301		44				
45		0 176		42	34	0 125	8
46	0 301		40				
47		0 301		37	44	0	−7
48	0 301		34				
49	0 477		66				
50		0 556		60	72	−0 079	−12
51	0 477		54				
52		0 477		50	54	0	−4
53	0 477		46				
54		0 301		45	30	0 176	15
55	0 477		44				
56		0 447		47	52	0 030	−5
57	0 477		50				
58		0 556		50	58	−0 079	−8
59	0 477		50				
60		0 415		47	50	0 062	−3
61	0 477		44				
62		0 301		44	38	0 176	6
63	0 477		44				

The values obtained in this way are given in table 5. The maximum per cent error is 4.5 with an average of 2.7.

The term, per cent standard error, in tables 4 and 5 is used to indicate comparative standard errors for the various assays. It is the standard error that would be obtained if all solutions assayed were of the same strength as the standard. The ratio, per cent error/per cent standard error, gives a value which

TABLE 3

Analysis of data in table 1

PARAMETER	FORMULA	VALUE
N	Number of doses of unknown	15
$S(X)$	Sum of X	0.510
$S(X^2)$	Sum of X^2	0.1247
$S(Y)$	Sum of Y	-26
$S(Y^2)$	Sum of Y^2	1,206
$S(XY)$	Sum of XY	9.667
$\bar{x}$	$S(X)/N$	0.034
$\bar{y}$	$S(Y)/N$	-1.73
(x^2)	$S(X^2) - \bar{x}S(X)$	0.1074
(y^2)	$S(Y^2) - \bar{y}S(Y)$	1,161
(xy)	$S(XY) - \bar{x}S(Y)$	10.551
b	$(xy)/(x^2)$	98.2
M	$\bar{x} - \bar{y}/b$	0.0516
Potency in per cent	100 (Antilog. M)	112.6%
Reduced (y^2)	$(y^2) - b(xy)$	124.9
s^2	Reduced $y^2/N - 2$	9.61
$V(X)$	$\dfrac{s^2}{b^2}\left\{\dfrac{\bar{y}^2}{b(xy)} + \dfrac{1}{N}\right\}$	0.000067
sM	$\sqrt{V(X)}$	0.0082
Standard error	$2.303sM(100)$ (Antilog. M)	2.13%

TABLE 4

Results of assays of "unknown" solutions

ASSAY NUMBER	NUMBER OF UNKNOWN DOSES	TRUE POTENCY	POTENCY FOUND	STANDARD ERROR	PER CENT ERROR	PER CENT STANDARD ERROR	% ERROR / % STD. ERROR
1-a	6	80	80.4	2.1	0.5	2.6	0.19
1-b	6	80	83.5	6.6	4.4	7.9	0.56
2-a	7	75	71.2	1.3	5.1	1.8	2.84
2-b	6	75	75.7	4.2	0.9	5.5	0.16
3-a	8	120	112.9	3.1	5.9	2.8	2.11
3-b	9	120	119.5	5.4	0.4	4.6	0.09
4-a	8	100	105.2	8.2	5.2	7.8	0.67
4-b	7	100	102.0	5.8	2.0	5.7	0.35
5-a	8	110	113.7	4.8	3.4	4.2	0.81
5-b	7	110	117.6	4.7	6.9	4.0	1.71
6-a	8	110	110.5	3.5	0.5	3.2	0.16
6-b	7	110	114.6	1.6	4.2	1.4	3.00
7-a	8	90	90.5	7.1	0.6	7.8	0.08
7-b	7	90	87.8	3.0	2.4	3.5	0.69
8-a	8	80	79.3	2.5	0.9	3.1	0.29
8-b	7	80	86.4	4.1	8.0	4.7	1.70
Mean					3.2	4.4	

shows how great the actual error is compared with the standard error This value may be compared with the values in Fisher's (9) t table (with $n = $ N-2) to determine whether the standard errors provide a valid measure of the reliability of individual assays In table 5 half of the values should be less than 0 71 to 0 74 ($P = 0 5$) Actually 10 out of 16 are less than 0 7 19 values out of 20 should be less than 2 37 to 2 78 ($P = 0 05$) 14 out of 16 are less than 2 2 Likewise, 99 values out of 100 should be less than 3 5 to 4 6 ($P = 0 01$) All values are actually less than 3 1 None of these values in table 5 exceeds the tabulated value for $P = 0 05$ The standard errors therefore appear to have their usual significance as a measure of the reliability of individual assays

The method of assay described has been used as an aid in the standardization of the oxytocic activity of posterior pituitary preparations 18 samples have been assayed by this method and 17 of them also by the U S P (10) uterine

TABLE 5

Results of assays* of "unknown" solutions

ASSAY NUMBER	NUMBER OF UNKNOWN DOSES	TRUE POTENCY	POTENCY FOUND	STANDARD ERROR	PER CENT ERROR	PER CENT STANDARD ERROR	% ERROR / % STD ERROR
1	12	80	82 4	1 2	3 0	1 4	2 14
2	13	75	73 2	1 9	2 4	2 5	0 96
3	17	120	116 4	3 4	3 0	3 0	1 00
4	15	100	103 3	6 1	3 3	5 9	0 56
5	15	110	115 0	3 2	4 5	2 7	1 67
6	15	110	112 6	2 1	2 4	1 9	1 14
7	15	90	89 3	3 6	0 8	4 0	0 20
8	15	80	81 8	2 3	2 3	2 8	0 82
Mean					2 7	3 0	

* Same assays as in table 4 but each pair (a and b) combined and calculated as one assay

method The usual procedure has been to first assay a preparation by the chicken method and then to run a U S P assay—the relative dosage of standard and unknown being the value obtained by the chicken assay Two preparations (1 and 2, table 6) were assayed first by the U S P method and later by the chicken method The results of the assays of the 18 preparations are shown in table 6 Results by both methods agree within the limits of error of the two methods as would be expected from the study by Smith (3) The limits of error are rather narrow for the chicken assays as indicated by the standard errors The U S P method does not include an estimate of the standard error but evidence of potency to within ±20 per cent of the potency required is acceptable In spite of the rather rigid specifications of the U S P method considerable is left to the experience and judgement of the assayist in estimating the reliability of individual assays The author believes the results of the U S P

assays in table 6 to be accurate to within ±10 per cent although the possibility of an occasional error as high as ±20 per cent cannot be discounted. Some of the results are undoubtedly more reliable than others but owing to the lack of suitable means for making an objective estimate of the reliability of individual assays all of the results must be regarded as subject to an error of about ±20 per cent.

Two uterine assays (on preparations 7 and 16) were continued according to the experimental design employed for the blood pressure assays. These assays

TABLE 6

Assays of posterior pituitary samples

PREPARATION NUMBER	TYPE OF PREPARATION	U.S.P. METHOD	CHICKEN METHOD			
		Potency found	Potency found	Standard error	% Standard error	Number of unknown doses
1	liquid	100*	101.1	2.5	2.5	25
2	liquid	110*	114.1	5.2	4.6	11
3	powder	100	103.2	2.1	2.0	15
4	powder	77	76.4	1.8	2.4	15
5	powder	95	94.2	3.7	3.9	15
6	powder	94	94.2	1.7	1.8	15
7	powder	47 ± 2.7**	46.5	0.9	1.9	15
8	powder	50	53.6	1.6	3.0	23
9	powder	110	107.9	3.8	3.5	15
10	powder	71	71.1	3.2	4.5	15
11	liquid	104	104.7	2.8	2 7	15
12	liquid	98	96 3	1.8	1.9	15
13	liquid		128.7	2.8	2 2	15
14	powder	77	77.6	2.4	3.1	14
15	powder	100	95.0	4.4	4.6	14
16	liquid	133 ± 4.5**	150.7	2.1	1.4	16
17	powder	50	49.8	2.1	4.2	10
18	powder	100	99.4	3.2	3 2	10
Mean					3 0	

* U.S.P. results obtained before chicken assay was run.

** Standard errors. The uterine assay was continued so that data were obtained and interpreted as for the chicken method.

each involved 6 doses of unknown and 7 of standard and the results were interpreted as previously indicated for the chicken blood pressure method except that the response for the uterine method was the height of the record produced by contraction of the uterine strip. The results were precisely the same as for the U.S.P. method but are presumably more reliable as indicated by the small standard errors for these results. A more extensive study of the applicability of Vos' (8) experimental design to the uterine method for the assay of posterior pituitary is under way. The chief difficulty here is in obtaining suitable uterine strips. Indications are that such a method cannot possibly surpass the chicken blood pressure method described here in simplicity, rapidity, economy or ac-

curacy Such a method would, however, provide a more suitable means of distinguishing differences in results by the uterine and blood pressure methods of assay In the assays of preparation 16, table 6, for instance, there was an apparently distinguishable difference in potency by the two methods The uterine assay was continued as previously indicated so that a more reliable result was obtained and the standard error could be calculated The result by each method has a standard error then and a formula for significant difference may be applied Application of the formula for significant difference given by Burn (11) results in a value of 3 57 Reference to Fisher's (9) t table shows that this value exceeds the 1 per cent level of probability ($P = 0.01$) This means that there is less than one chance in a hundred that the difference is due to experimental error This suggests that the chicken blood pressure method cannot always be relied on to give precisely the same result as the U S P uterine method in the assay of the oxytocic activity of posterior pituitary

DISCUSSION Application of the experimental design and method of interpretation used by Vos (8) for the biological assay of ergonovine on rabbit uterus to the chicken blood pressure test reported by Coon (2), as herein described provides a simple, rapid, economical, and accurate method for the biological assay of the oxytocic activity of posterior pituitary extracts The method is simple because anyone familiar with manometric methods of recording blood pressure can master it The calculations involve little more than simple arithmetic since the required formulae are provided by Vos (8) and their application to the present method is shown herein An assay comprising 15 doses of unknown and 16 doses of standard is completed in little over one and one half hours An assay sufficiently accurate for most purposes, comprising seven doses of unknown and eight doses of standard, is completed in about forty five minutes It is usually possible to anesthetize a rooster, prepare it for recording blood pressure and then to run three or four complete assays (about 31 doses each) in a single day on the same rooster Rarely it is necessary to discard a rooster because of low blood pressure, rapid development of tachyphylaxis, or low sensitivity to the drug

The precision of the method is greater than for any method previously reported This is demonstrated by the internal evidence in each assay and by the results from the assay of solutions of known potency

A practical feature of the method may be pointed out In the assay of a posterior pituitary powder for instance, separately prepared solutions may be used for each half of the assay With little additional work the data from each half may then be calculated separately to give fairly accurate results for each solution The significance of any difference may be determined mathematically since each result would have its standard error This provides a good check against errors in preparing the solutions If no significant difference occurs the result may be computed from the entire data to obtain the ultimate in precision

SUMMARY AND CONCLUSIONS

1 A simple, rapid, economical method is presented for the biological assay of the oxytocic activity of posterior pituitary solutions

2. Results and reliability of individual assays are computed mathematically.

3. The method is more accurate than any previously reported. The maximum error obtained in the assay of solutions of "unknown" potency was 4.5 per cent with an average error of 2.7 per cent.

4. Further evidence as to the agreement between results by the chicken blood pressure method and the official uterine method was obtained. One sample showed a difference in potency by the two methods which was shown to be highly significant statistically but the difference was only 12 per cent.

5. If the chicken blood pressure test replaces the now official uterine method for the biological assay of the oxytocic activity of posterior pituitary solutions it should be adopted in a form that would permit the experimental design and method of interpretation used in this study.

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STUDIES ON ANTIMALARIAL DRUGS

The Excretion of Atabrine in the Urine of the Human Subject[1]

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Experiments on the metabolism of atabrine in dogs receiving six oral doses a week, have shown that the maximum rate of excretion in the urine and feces is attained within one or two weeks, and does not exceed 4% of the daily dose (1) Because of the extensive present-day use of atabrine, these studies were undertaken to determine whether the rate and magnitude of excretion in the human subject parallels that of the dog

Recent work on the relative specificity of analytical methods has shown certain atabrine metabolites to have solubility and fluorescent characteristics which would reduce the specificity of the older methods (1, 2) Extraction of the sample with a suitable organic solvent and followed by alkali washing results in a relatively pure extract Assay of this extract by the use of a sensitive photo-fluorometer with specific light filters, gives results more nearly approaching the true atabrine value than with any other available method (1)

EXPERIMENTAL Four subjects, F K O , female, age 28, M S , male, age 30, E H D , male, age 28, and F E K , male, age 30, were given atabrine dihydrochloride (Winthrop Chemical Company) in tablet form with the evening meal in dosages of 100 mgm per day for 45 consecutive days Twenty-four hour urine collections were made for the two preceding days and at various intervals during and after the period of treatment Aliquots were analysed for atabrine by the method previously described (1), using the B_2PC-2 filters and making the readings in ammoniacal 50% alcohol The extracts of the control urines showed no detectable fluorescence

RESULTS AND DISCUSSION All subjects reported mild symptoms attributable to the atabrine administration which were difficult to evaluate or describe They consisted for the most part of a mild diarrhea, slight general malaise and frequent headaches Although none of these symptoms could be considered a definite contraindication for the drug, the experience was not a pleasant one, and the experiment was discontinued because of the general uncomfortable feeling the drug induced, together with the conspicuous yellow pigmentation that developed during the last two or three weeks of administration Two other volunteers discontinued the experiment after the second dose of atabrine due to rather severe allergic-like reactions in which a profuse rhinorrhea was one of the predominant symptoms

The results of the urinary analyses are given in the accompanying figure. There is fairly good agreement between the several subjects, although M. S. tended to show somewhat higher values than the others. This subject also
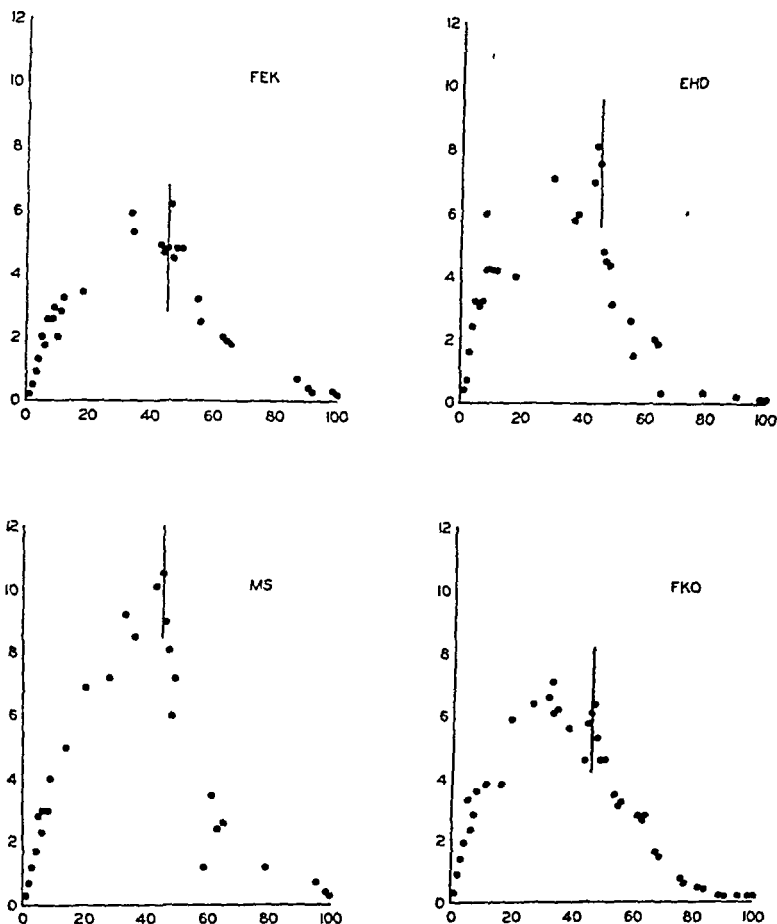
FIG. 1. EXCRETION OF ATABRINE IN 24 HOUR URINE SAMPLES IN FOUR HUMAN SUBJECTS

The ordinates represent the amount of atabrine excreted expressed as percent of the daily dose. The abscissa represents time in days. Atabrine dihydrochloride (100 mgm.) was given once a day for the first 45 days.

exhibited the most marked pigmentation, which suggests that this subject was not able to degrade the atabrine as readily as the others.

In all cases, the daily urinary excretion of atabrine increased gradually for the first three or four weeks. There is some indication of a stabilization of the per-cent of the daily dose excreted after this interval, although the daily values for

M S continued to increase until the drug was discontinued After the last dose, the urinary excretion began to drop but a significant amount was excreted even on the last day urine samples were taken, 55 days after the last dose

SUMMARY

The daily urinary excretion of atabrine was studied in four subjects receiving 100 mgm per day for 45 days The amount excreted rose slowly throughout the experiment but in no case exceeded 11% of the daily dose Fifty five days after the last dose appreciable amounts of atabrine were present in the urine All subjects showed mild toxic symptoms

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STUDIES CONCERNING THE ABSORPTION AND DETOXIFICATION OF ANESTHETIC STEROIDS

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All hormonally active steroids, and even some steroid compounds which possess no hormonal activity, have definite anesthetic properties if they are administered by the intravenous or intraperitoneal route. This effect is not species specific since it has been demonstrated in the fish, bird, cat, guinea pig, mouse, rabbit and monkey (1–10). It was found, furthermore, that many of these compounds exhibit their anesthetic activity even after oral administration (11) and that bile does not appear to play an important role in the mechanism of their absorption from the intestin (12). In order to obtain the anesthetic effect, it is apparently essential to flood the organism suddenly with a high concentration of these steroids. A good deal of attention has also been paid to the fate of the anesthetic steroids in the body and more particularly to the mechanism of their detoxification within the organism. It was found that hypophysectomy, adrenalectomy and especially partial hepatectomy increase the sensitivity of the rat to the anesthetic effect of desoxycorticosterone acetate and progesterone. From this we concluded that the pituitary, adrenals and the liver are in some way involved in the detoxification of the steroids and that in the absence of one of the above-mentioned glands the sensitivity to the anesthetic effect increases because the hormone remains in an active form in the organism for a longer period than is usually the case (7).

The anesthetic effect of the steroids appears to be a particularly suitable indicator of activity in studies concerning their absorption rate because anesthesia is only produced if the compounds are rapidly absorbed. Hence from the anesthetic effect of a given quantity of a steroid we may gauge the speed with which it is absorbed. The narcotic action may also be used in the study of hepatic detoxification since partially hepatectomized animals are more sensitive to this effect than intact controls. In this manner it is possible to estimate the degree of hepatic detoxification by the decrease in the anesthetically-effective dose conditioned by partial ablation of the liver. The hormonal properties of steroids are less suitable for this type of test because a longer period of treatment is necessary to obtain them and after hepatectomy the remnant of the hepatic tissue undergoes such active regeneration that its function rapidly returns to normal.

In view of these considerations we decided to investigate the absorption rate and hepatic detoxification of some of the more important steroids using the anesthetic effect as an indicator of their potency.

EXPERIMENTAL. All our experiments were performed on female albino rats weighing 50 g. on the average (range 40–60 g.). The study of each steroid compound was performed

on three groups of rats   Group I was partially hepatectomized and received the steroids in oil solution by intraperitoneal injections (concentration 20 mg per cc), group II which was also partially hepatectomized, received finely ground crystal suspensions of the steroids administered orally through a stomach tube the total dose always being suspended in 1 cc of water, group III consisted of intact rats receiving the steroids orally in the same manner as the animals of group II   Oil had to be used as a vehicle in group I and water in groups II and III because our previous experiments showed that from the peritoneum the compounds are more readily absorbed when given in true solution—and generally speaking these steroids are much more soluble in oil than in water—while from the intestine even crystals are well absorbed but oil interferes with the absorption mechanism   In each case varying doses of the steroids were tested in order to establish the anesthetic unit as previously defined (5) that is the dose just sufficient to cause loss of the righting reflex in 4 out of 6 rats   Since fasting rats respond more uniformly to orally administered steroids than fed animals all the experiments of this series were performed on rats after 24 hours of fasting   Otherwise the conditions of experimentation especially the technic of hepatectomy and the interval between the operation and the administration of the steroids were the same as in our earlier work on this subject (5)   It will be noted however that since fasting sensitizes the rat to the anesthetic effect of the steroids the anesthetic rat units recorded in this paper tend to be somewhat lower than those previously reported   In the case of steroids which proved ineffective at all dose levels the highest dose tested is listed in brackets

Our results are summarized in table 1 in which the systematic chemical name (for terminology see Selye 13) as well as, in brackets, the trivial name of each compound are listed together with the melting point of our sample and the number of rats used   Depending upon the number of experiments required to determine the threshold anesthetic dose, the total number of experiments performed with each compound varied considerably   However, it would take a great deal of space to report each experiment individually and hence they are combined in the table and only the total number of animals is listed to give a general idea of the size of the series used   The anesthetic rat unit is recorded for each of the three groups, as well as the ratio between the unit in groups I and II, and II and III respectively   The ratio I II visualizes the comparative effectiveness of intraperitoneal and oral administration of the steroids in the partially hepatectomized rat   From this it will be seen that, under the conditions of our experiments, the acetate and ½ succinate of desoxycorticosterone, pregnanedione and progesterone have an absorption ratio of 1 1, that is to say, they are equally well absorbed from the peritoneum and the intestine   Methyl testosterone has a ratio of 1 1 5, indicating a somewhat better absorption from the peritoneum than from the alimentary tract   All other steroids listed in the table proved to be even less readily absorbed when given by mouth   These figures clearly indicate that there are considerable differences among the steroids as regards their absorbability from the gastro-intestinal tract and that generally speaking the most potent anesthetics also proved most readily absorbable following oral administration   The results concerning testosterone and methyl testosterone deserve special consideration inasmuch as the latter compound, though hormonally more active than the former, proved less active as an anesthetic in hepatectomized animals   This is in agreement with the interpretation (12) according to which—contrary to common belief—the great oral effectiveness of methyl

testosterone is not due to its ready absorbability from the gastro-intestinal tract, but on the contrary to the fact that its absorption from the intestine is slow. This is detrimental for the production of anesthesia but beneficial for hormonal effectiveness. As judged by our figures the delay in absorption rate is even greater in the case of intraperitoneal than in the case of oral administration.

TABLE 1

Absorption and detoxification ratios of anesthetic steroids

NUMBER OF COMPOUND	COMPOUND	M.P.	NUMBER OF RATS	GROUP I, HEPATECTOMIZED I.P.	RATIO I:II	GROUP II, HEPATECTOMIZED P.O.	RATIO II:III	GROUP III, NORMAL P.O.
		°C						
I	17(α) - [1 - keto - 2 - hydroxyethyl] - Δ^4 - *androstene* - 3 - one-17²-acid succinate (Desoxycorticosterone ½ succinate)	192~200	30	0.6	1:1	0.6	1:5	3.0
II	17(α) - [1 - ketoethyl] - *etiocholane*-3-one (Pregnanedione)	120~122	22	0.8	1:1	0.8	1:5	4.0
III	17(α) - [1 - keto - 2 - hydroxyethyl] - Δ^4 - *androstene* - 3 - one - 17² - acetate (Desoxycorticosterone acetate)	156~157	53	1.0	1:1	1.0	1:4	4.0
IV	17(α) - [1 - ketoethyl] - Δ^4 - *androstene*-3-one (Progesterone)	122	82	1.0	1:1	1.0	1:4	4.0
V	17(α) - [1 - keto - 2 - hydroxyethyl] - Δ^5 - *androstene* - 3(β) ol - 17² - acetate (Acetoxypregnenolone)	185~186	75	1.2	1:2.5	3.0	1:7	20.0
VI	Δ^4 - *androstene* - 3 - one - 17(α) - ol (Testosterone)	153~154	77	3.0	1:2.2	7.0	1:7	50.0
VII	17(β) - methyl - Δ^4 - *androstene*-3-one-17(α)-ol(Methyl-testosterone)	164~165	31	6.0	1:1.5	9.0	1:3.5	32.0
VIII	17(α) - [1 - ketoethyl] - Δ^5 - *androstene*-3(β)-ol (Pregnenolone)	186~187	21	12.0	1:6	70.0	1:>3	Inactive (180)
IX	$\Delta^{1,3,5:10}$ - *estratriene* - 3,17(α) - diol (α-estradiol)	176~177	36	15.0	1:5.5	85.0	1:>3	Inactive (180)
X	17(β) - ethynyl - Δ^4 - *androstene* - 3 - one - 17(α) - ol (Ethynyltestosterone)	267~268	23	20.0	1:>3	Inactive (60)		Inactive (180)

Comparing group II with group III we obtain the "hepatic detoxification ratio." Since our experiments were performed on rats which were still in possession of about 25% of their liver tissue, these ratios should obviously not be interpretated as indicating the actual percentage of the various compounds which is detoxified by the liver, as opposed to the total amount detoxified in the body. The ratio merely gives an estimate of the extent to which the same degree of

hepatic insufficiency interferes with the detoxification of different steroids   It
will be kept in mind that the steroids were given orally in aqueous suspension
both in group II and III and hence the only difference between these two groups
was the absence of the major part of the liver tissue in the former series   The
table indicates that in the case of most steroids the hepatic detoxification ratio
is in the vicinity of 1 5 or 1 4, hence, we may say that most steroids examined
by us become 4 to 5 times more active in the partially hepatectomized rat than
they are in the intact animal   It is noteworthy, however, that the lowest ratio
1 3 5 was observed for methyl testosterone, while testosterone was in the group
having the highest ratio of 1 7   That is to say, the hepatic detoxification of
testosterone is apparently greatly inhibited by methylation   This may help
to explain the greater oral effectiveness of methyl testosterone as compared to
testosterone when both are given by mouth to the intact rat   The fact that in
group II, and even more markedly in group I, methyl testosterone proves con-
siderably less active than testosterone, may be due to the above mentioned
delay in the absorption rate conditioned by methylation   Hence it is rather
probable that the increase in the hormonal effectiveness of testosterone induced
by methylation at C_1 is due partly to a more favorable (slower) absorption rate
and partly to a protection against hepatic detoxification

SUMMARY

Experiments on immature female albino rats revealed that there are great
differences in the relative absorption rate of steroid hormones and hormone
derivatives from the peritoneum and the intestine   However, progesterone,
the acetate and succinate of desoxycorticosterone and pregnanedione, are equally
well absorbed from the peritoneum and the intestine under the conditions of
our experiments

Hepatectomy was shown to sensitize the organism to the anesthetic action
of all steroids examined   In the case of most of these compounds ablation of
75% of the liver tissue raised the sensitivity of the organism four to five times
above that of intact controls but apparently the different steroids are not equally
well detoxified by the liver

Methyl testosterone is less active as an anesthetic than testosterone when
assayed on partially hepatectomized rats   This may be due to the delay in the
speed of absorption occasioned by methylation   a delay which is advantageous
for hormonal, but detrimental for anesthetic activity   In the case of oral ad-
ministration to intact rats, methyl-testosterone is more active than testosterone,
even as an anesthetic, apparently because the much greater hepatic detoxifica-
tion of testosterone over-compensates for its more advantageous (rapid) ab-
sorption

excepting the pregnanedione and desoxycorticosterone $\frac{1}{2}$ succinate which were kindly supplied by Dr. R. D. H. Heard of the Department of Biochemistry of McGill University.

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STUDIES ON ANTIMALARIAL DRUGS

The Preparation and Properties of a Metabolic Derivative of Quinine[1]

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Quinine is converted *in vitro* by certain tissues to one or more substances which no longer have the solubility or fluorescent characteristics of quinine (1–4) This report deals with the isolation and crystallization of one of the products of this reaction and a description of some of its chemical and physical properties

Studies on the rate of disappearance of injected quinine from the tissues in various species (5 6), and observations on the enzymatic destruction of quinine *in vitro* (3, 4) showed that rabbit liver is remarkably active and hence it was used in this work With sheep liver, Lipkin (1) isolated a substance identified as quitenine and with guinea pig liver another product which he was unable to identify However, the compound to be described here differs in its chemical and physical properties from either of these substances and from all known derivatives of quinine

EXPERIMENTAL *Enzymatic conversion of quinine* Two kgm of fresh rabbit liver were ground and blended with eight liters of Ringer-Locke solution to which had been added an additional gram of glucose and of bicarbonate per liter Twenty-five grams of quinine hydrochloride were added, the mixture was stirred and aerated by a jet of air and was maintained at 38° by a water bath for five hours

Isolation of the quinine degradation product The reaction mixture was made basic by the addition of NH₄OH and extracted with an equal volume of n-butyl alcohol The alcoholic extract was diluted with an equal volume of petroleum ether and extracted several times with dilute H₂SO₄ The H₂SO₄ extract was made alkaline with KOH and extracted four times with ether to remove the quinine The KOH extracts were made acid with H₂SO₄ and then basic with NH₄OH The precipitate was filtered, washed with water, dissolved in alcohol, diluted with water and set aside to crystallize The filtrates were combined and extracted with n butyl alcohol as before to obtain a second crop of crystals The yield was 37%

Physical and Chemical Properties The crystals exhibited a change in birefringence at 150° and melted to a clear yellow oil at 251° (corr) No loss in weight occurred on drying for two hours at 100° under vacuum The molecular weight as determined by depression of the camphor m p was 370

The metabolite is very soluble in dilute acid or alcohol, slightly soluble in acetone, ethyl ether or dilute KOH, and relatively insoluble in water, dilute NH₄OH, petroleum ether or benzene. Butyl alcohol or chloroform extract the compound from dilute NH₄OH but not from dilute KOH.

DISCUSSION. There is evidence of marked differences in the ability of the liver of different species to produce the metabolic transformation product just described (3). A substance having similar solubility properties was isolated in small amounts from the urine of 10 human subjects who had received quinine sulfate by mouth but the quantity obtained was insufficient for identification. The quinine metabolite exhibits a small amount of fluorescence under the conditions of fluorimetric assay usually used for the determination of quinine (7,8). This fluorescence is not abolished by halogen salts, in contrast to the fluorescence of quinine. Since the metabolite is not extracted by ether from dilute sodium hydroxide or potassium hydroxide solutions, it does not appear in the final alkaloid extract obtained using our previously described method for quinine (8) and therefore does not cause falsely high readings with this method.

Detailed studies of the toxicity and antimalarial activity of this compound are under way. A report of studies being done elsewhere on its chemical structure will follow.

SUMMARY

A method for the isolation and crystallization of a newly described detoxification product of quinine has been presented.

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STUDIES ON THE DETOXICATION OF ORGANIC ARSENICAL COMPOUNDS

V ADDITIONAL DETOXICANTS FOR PENTAVALENT ARSENICALS

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Among its several important biological properties, p amino benzoic acid (hereinafter abbreviated PABA) possesses a remarkable capacity to protect rats against the lethal action of massive doses of such phenyl arsonic acid derivatives as 'Atoxyl', Acetarsone, Carbarsone, 'Tryparsamide', etc (1, 2, 3) It has also been found to be an effective detoxicant of the pentavalent antimonial drug, 'Stibosan' (4) and the trivalent arsenical drug, neoarsphenamine (5) On the other hand, it appears to have little prophylactic value against acutely lethal doses of the inorganic compounds of arsenic and antimony or against such trivalent compounds as tartar emetic, 'Mapharsen', or arsphenamine (5)

Inasmuch as the original discovery of the arsenic detoxicating property of PABA arose as an incidental finding from an experiment performed for an unrelated reason, it was considered likely that further search would reveal other substances of similar if not superior protective value and at the same time, perhaps, provide a clue to the mechanism underlying the detoxication process The results of protection experiments utilizing a number of compounds of the aromatic series are presented in this paper

The selection of compounds for trial was determined largely on a basis of their easy availability on the present open market as well as on certain aspects of their structural relationship to PABA

EXPERIMENTAL METHODS The conditions under which this investigation was pursued have been essentially those described in previous publications of this series Rats of a mixed stock weighing from 90 to 125 grams were treated at dosage rates roughly in proportion to their weight They were kept under observation for at least 10 days before the results of protection tests were finally recorded

As a basis for comparing the detoxicating values of the various substances subjected to test it was decided to utilize as tentative reference standards the extensive data on the toxicity of certain arsenicals and the protective effect of PABA that had accumulated from experiments previously completed in this laboratory It had been our experience that approximately 90 per cent of rats succumb within 10 days following the intraperitoneal injection of arsanilic acid (p amino phenyl arsonic acid) at the rate of 400 mgm /kg or of Carbarsone (p carbamino phenyl arsonic acid) at the rate of 1000 mgm /kg Up to that time not a single rat had been known to tolerate an injection of 450 mgm /kg of arsanilic acid or 1500 mgm /kg of Carbarsone All or nearly all of such animals would survive if, prior to the arsenical PABA at a rate of 750 mgm /kg was administered either by mouth or by intraperitoneal injection

Consequently in performing protection assays new compounds were injected at the rate of 750 mgm /kg providing that prior toxicity tests on a few rats had shown that this dose was well within the range of tolerance In order to observe a reasonable factor of safety

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it was found necessary to administer some compounds, e.g., *o*-hydroxy benzoic acid, *m*-nitrobenzoic acid, etc., by the oral route and at considerably lower dosage rates. With other compounds that are relatively nontoxic, e.g., nicotinic acid, the dose was elevated to 1000 mgm./kg. or more, in order to elicit the maximum detoxicating potential. Compounds soluble in water or weak alkali were injected in 10 to 20 per cent solution. Water insoluble compounds were administered orally, either in alcoholic solution (e.g., acetanilid) or in gum acacia suspension (e.g., tyrosine). As already indicated, the detoxicating dose was exhibited usually from 5 to 15 minutes prior to the arsenical injection. It may be noted that we have been unable to demonstrate any benefit deriving from repeated injections of the detoxicant after the arsenical has already been given.

DISCUSSION. Those conversant with the rather rigid technical requirements for establishing acceptably exact standards for the toxicity of arsenical drugs will appreciate the even greater difficulty of setting up experiments extensive enough as to provide statistically adequate criteria of the relative detoxicating capacities of a large and diverse series of compounds.

To the extent that the detoxication trials whose results are tabulated above were not conducted with random samples of a homogeneous population of rats, all the tests being performed substantially at one time, it would be unwarranted to endow the "percentage survival" figures that have emerged from these tests with precise quantitative values. However, assays with certain related compounds (e.g., nicotinic acid and nicotinamide, benzoic acid and benzaldehyde) were actually run as parallel experiments and, as may be inferred from the tabulation, many of the assays were repeated on several occasions to substantiate the results and to provide assurance as to their broad reproducibility. For these and other reasons it appears legitimate to consider the results of detoxication experiments as affording at least a rough measure of the relative detoxicating potentials of the various compounds tested at the dosage levels specified. On the basis of this assumption, the following general conclusions are in our opinion warranted by the data.

The ability of PABA to counteract the lethal consequence of particular massive doses of phenyl arsonic acid and derivatives[1] is obviously not a function of its specific chemical constitution. Detoxicating properties of comparable magnitude are possessed not only by its ortho- and meta-isomers but also by such nitro-, hydroxy-, and methyl-analogues as are not *per se* highly toxic to the rat. It is also apparent that the simple unsubstituted aromatic acids of the benzoic series (e.g., phenyl acetic and phenyl propionic acids) are equally effective detoxicants. In addition to homocyclic compounds of the benzene ring series, we have found pyridine 3-carboxylic (= nicotinic) acid to be a potent protective substance. In contrast, proline (pyrrolidine-2-carboxylic acid) appears to be ineffective.

Substitution by hydroxyl- or nitro-radicals in the benzene ring of aromatic

[1] Several series of detoxication tests have been run against 'Tryparsamide' (Sodium N phenyl glycine amido *p*-arsonate) at the dose rate of 4500 mgm./kg. and phenyl arsonic acid at the dose rate of 60 mgm./kg. The detoxication records of the various compounds substantially run parallel to those obtained against Carbarsone and arsanilic acid. They are omitted from the tabulated results because of their incompleteness.

TABLE 1

Summary of results of detoxication assays showing percentage survival of rats

Figures in parentheses represent the collated number of rats involved in the percentage calculations

PRESUMPTIVE DETOXICANT	DOSE (MGM/KG) AND ROUTE	ARSANILIC ACID		CARBARSONE	
		400 mgm/kg	450 mgm/kg.	1000 mgm/kg	1500 mgm/kg
	Controls	14 6 (150)	8 7 (92)	10 8 (175)	1 0 (95)
p aminobenzoic acid H_2N⟨⟩COOH	750 i p	87 (115)	90 (20)	94 (80)	97 (40)
m-aminobenzoic acid H_2N⟨⟩COOH	750 i p	75 (20)	80 (10)	100 (10)	70 (10)
o-aminobenzoic acid ⟨⟩^{NH_2}COOH	750 i p.	80 (20)	90 (10)		100 (10)
3,5-diamino benzoic acid H_2N⟨⟩COOH H_2N	375 i p.	10 (10)		7 (15)	
p chlorobenzoic acid Cl⟨⟩COOH	600 i p.	80 (10)	40 (10)	90 (10)	30 (10)
p-nitrobenzoic acid O_2N⟨⟩COOH	750 i p.	75 (20)	90 (10)	100 (10)	100 (10)
m-nitrobenzoic acid O_2N⟨⟩COOH	400 i p.	90 (10)	90 (10)	100 (10)	90 (10)
p-hydroxybenzoic acid HO⟨⟩COOH	750 i p.	80 (20)	90 (10)	100 (10)	100 (10)
salicylic acid ⟨⟩^{OH}COOH	500 oral	40 (10)		50 (10)	
o-acetyl salicylic acid ⟨⟩^{OOCCH_3}COOH	500 oral	90 (10)	50 (10)	100 (10)	50 (10)
methyl p amino benzoate H_2N⟨⟩COOCH_3	750 oral			0 (10)	

TABLE 1—*Continued*
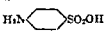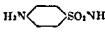
PRESUMPTIVE DETOXICANT	DOSE (MGM./KG.) AND ROUTE	ARSANILIC ACID		CARBARSONE	
		400 mgm./kg.	450 mgm./kg.	1000 mgm./kg.	1500 mgm./kg.
acetanilid ⬡NHCOCH₃	750 oral	75 (20)	10 (10)	87 (15)	20 (20)
mandelic acid ⬡CHOHCOOH	750 i.p.	70 (10)	30 (10)	50 (10)	30 (10)
dl-phenylalanine ⬡CH₂CHNH₂COOH	1500 oral	40 (10)		0 (5)	
dl-p-hydroxyphenylalanine HO⬡CH₂CHNH₂COOH	750 oral	0 (15)		30 (10)	
benzaldehyde (bisulfite) ⬡CHO·NaH·SO₃	750 oral	60 (10)	85 (20)	90 (20)	60 (15)
benzoic acid ⬡COOH	750 i.p.	96 (30)	80 (30)	92 (40)	95 (20)
p-toluic acid H₃C⬡COOH	750 i.p.		95 (20)		100 (10)
phenyl acetic acid ⬡CH₂COOH	750 i.p.	100 (30)	90 (20)	100 (30)	100 (20)
p-amino phenyl acetic acid H₂N⬡CH₂COOH	750 i.p.	100 (15)	70 (10)	90 (10)	65 (20)
phenyl propionic acid ⬡CH₂CH₂COOH	500 i.p.	90 (10)	70 (10)	100 (5)	100 (10)
benzyl succinate ⬡CH₂OOCCH₂CH₂COOH	750 i.p.		95 (20)		95 (20)
benzoyl glycine ⬡CONHCH₂COOH	750 i.p.		80 (15)		60 (20)
p-amino benzoyl glycine H₂N⬡CONHCH₂COOH	750 i.p.	100 (10)	85 (20)	100 (10)	80 (20)
N-phenyl glycine ⬡NHCH₂COOH	750 i.p.		100 (10)	100 (10)	90 (10)
nicotinic acid ⬡ᴺCOOH	1000 i.p.	95 (20)	100 (10)	100 (10)	60 (10)

TABLE 1—Concluded

PRESUMPTIVE DETOXICANT	DOSE (MGM/KG) AND ROUTE	ARSANILIC ACID		CARBARSONE	
		400 mgm/kg	450 mgm/kg	1000 mgm/kg	1500 mgm/kg
nicotinamide	1000 i p	~0 (10)	13 (15)	28 (25)	
proline	750 i p	20 (10)		10 (10)	
sulfanilic acid	750 i.p.	30 (10)	30 (~0)	25 (20)	
sulfanilamide	~50 oral	20 (15)		0 (10)	

acids does not significantly impair detoxicating power unless (as in the *ortho* position isomers) the intrinsic toxicity of the compound is thereby enhanced Thus salicylic acid is much less effective than *p* hydroxybenzoic acid, while considerable restoration of detoxicating capacity is shown by its less toxic derivative, acetyl salicylic acid The halogen derivatives, as represented by *p* chlorobenzoic acid, also show reduced detoxicating power The only *di* substituted compound tested, namely 3,5 diaminobenzoic acid, was too toxic to show detoxicating properties Esterification apparently annuls the detoxicating value of the aromatic acids, on the other hand little if any reduction occurs when an alkyl radical is introduced into the nuclear ring (compare methyl *p* aminobenzoate with *p* toluic acid, acetanilid with N-phenyl glycine) Sulfanilic acid and its derivative, sulfanilamide, are, judged by the results of our tests, essentially without detoxicating value

From these considerations of the aromatic compounds we may conclude that compounds with a free carboxyl (*rel* carbonyl group, as in benzaldehyde) attached to a closed 5 or 6 carbon ring nucleus are most conducive to detoxication The property persists even if the carboxyl is remotely attached to the nuclear ring as in phenyl glycine or benzyl succinate

While the effect of various changes in the side chain carrying the carboxyl has not been extensively explored, it is evident that the introduction of an amino- or hydroxyl group in the alpha position (as, for example, in phenyl alanine, tyrosine or mandelic acid) is associated with substantially reduced detoxicating power This may well be due to the amphoteric nature of the compound with a resultant reduction in aqueous solubility Such compounds must necessarily be administered by mouth and may not be substantially absorbed from the alimentary tract Transformation of the carboxyl into an amide also significantly reduces the detoxicating efficacy of a compound as exemplified by the results

secured with nicotinic acid and nicotinamide. The introduction of an amino-group into the nuclear ring may or may not be influential. It consistently reduced the detoxicating potential of phenyl acetic acid, but it does not appear to affect the results significantly with benzoic acid and p-aminobenzoic acid or with benzoyl glycine and p-aminobenzoyl glycine.

In conclusion, we are inclined to believe that the experiments here reported, together with those already published in these studies, are sufficient to render implausible the idea that detoxication of the pentavalent aromatic arsenicals is a result of direct chemical combination between the varied toxicant and detoxicant compounds with the formation of relatively inert products. By the same token we may reject the inference that the phenomenon is to be explained on the basis of a competition for essential metabolites or enzyme substrates that may occur between compounds with a high degree of structural similarity. Whether by virtue of a common property the various effective detoxicants are able to induce in the rat a physiological change that reduces the animal's susceptibility to acute poisoning by overdosage with diverse pentavalent arsenical and antimonial drugs is an attractive subject for future investigation.

SUMMARY

Continuing studies previously reported, it is here shown that many aromatic compounds have the property of inhibiting the lethal consequence of massive doses (ca. L.D. 90+) of pentavalent aromatic arsenicals in rats. In addition to the three isomeric forms of aminobenzoic acid, a high order of protection is conferred by the hydroxy- and nitro-analogues of PABA, as well as by other substituted compounds which are readily soluble and not in themselves highly toxic to the test animal. Inasmuch as benzoic, phenyl acetic, and phenyl propionic acids are all highly effective, it appears that a high degree of structural similarity between detoxicant and toxicant is not an essential of the mechanism underlying the detoxication phenomenon.

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INHIBITION OF HISTAMINE EFFECTS BY COMPOUNDS OF HISTAMINE, HISTIDINE, AND ARGININE

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In 1937, Edlbacher, Jucker and Baur (1) have shown that several amino acids (arginine, histidine and cysteine) counteract the effects of histamine upon the isolated guinea pig gut   In 1939, Ackermann and Wasmuth (2) were able to fully confirm the results of the previous investigators and have shown that even in combination with other amino acids forming complex protein molecules (as clupein which contains 80% of arginine) arginine inhibits the effects of histamine upon smooth muscle structures   As guanidine and derivatives display like inhibitory action, Ackermann (3) concluded that the guanidyl radical present in the arginine molecule would be responsible for the anti histamine effect   On the basis of those findings, Ackermann assumed that the $=NH$ imine group present in the arginine molecule and in the imidazol ring of histidine, would compete with the like $=NH$ group of the imidazol ring of histamine   According to this theory, this imine radical would be the anchoring group for histamine to fix upon the chemical receptors of smooth musculature   If a considerable excess of either arginine or histidine is previously added to the perfusing bath, histamine would have no chance to combine with the saturated chemical receptors and produce its characteristic stimulant effect   The interest of this theory is that it suggests a definite separation between the toxic effects of histamine (which depends on the NH_2 amine radical) and its anchoring ability to the cell receptors since the amino acids which display a like capacity to combine with those receptors are entirely devoid of pharmacological activity

The possibility to separate those two functions of histamine, would enable one to block the grouping that is responsible for the pharmacological effect and at the same time to keep unchanged the capacity for histamine to anchor upon the chemical receptors of the cells   This might provide a class of histamine derivatives as effective in inhibiting the histamine effects, as arginine or histidine themselves   Such conditions were fulfilled by the five histamine compounds which we have recently been able to synthesize at Dr Bergmann's laboratory   at the Rockefeller Institute—the acetyl dehydrophenylalanyl histamine   the acetyl d l phenylalalnyl histamine,   the benzoyl l, tyrosyl histamine   the carbobenzoxy l, tyrosyl histamine and the carbobenzoxy l leucyl histamine   The pharmacological study previously presented (5), has shown that they are entirely pharmacologically inactive upon the isolated guinea pig gut the human skin and the circulatory apparatus of the cat   This shows that blocking the NH_2 amine radical through chemical combination with the carboxyl groups of amino acids would destroy the pharmacological activity of histamine

On the other hand, the fact that the imine radical =NH of the imidazol ring is kept free in those compounds would induce one to verify whether those compounds display any competitive effect upon histamine itself.

The experiments reported in this paper lends full support to Ackermann's theory that the anchoring group of histamine is the imine =NH radical of the imidazol ring. The convenience to extend further our knowledge on the antihistamine effect produced by amino acids and derivatives led us to try several arginine compounds as benzoyl-*l*,arginine-amide, benzoyl-*l*,arginine and hippuryl-nitro,*l*,arginine, some of them being several times as powerful as arginine itself.

EXPERIMENTAL. The fragments of isolated guinea pig ileum were suspended in a 4 cc. perfusion bath, in a Dale's apparatus. No atropine was added. The testing solutions were added through common tuberculin syringes graduated in 0.01 cc.

The compounds to be tested were accurately weighed and solutions in saline were made. Most of them dissolved very well and were tried in concentrations amounting to M/10 to M/20. Benzoyl-*l*,tyrosyl-histamine was dissolved after addition of an equivalent of HCl and the solution (M/50) was carefully neutralized just before addition to the perfusing bath. Benzoyl-*l*,arginine and hippuryl-nitro,*l*,arginine were used in rather dilute solutions (up to M/250) made up in hot saline. Just before using, the solutions were cooled to 37° and added to the perfusing bath. As a rule, a faint precipitate formed which would introduce a slight incorrection in our figures for those two compounds. Acetyl-dehydrophenylalanyl-histamine HCl, acetyl-*d*,*l*,phenylalanyl-histamine HCl, arginine mono-hydrochloride, benzoyl-*l*,arginine-amide and histidine monohydrochloride were easily dissolved at the concentrations of M/10 to M/20. Consequently, the results obtained with those compounds are strictly quantitative and can be used for a thorough discussion of the relationship between chemical composition and anti-histamine effect.

RESULTS. *Experiments on the isolated guinea pig gut.* To establish our reasoning on solid grounds it has been necessary to devise a technique to work on quantitative basis. This goal was attained by determining the exact amount of substance necessary to extinguish the stimulant effect of known amounts of histamine. Thus, an arbitrary small amount of each compound was added to the bath and 30″ allowed to elapse before adding the proper amount of histamine. After washing out with new Tyrode solution, a greater amount of the substance and (after 30″) the same amount of histamine was added. This operation was continued until total extinction of the histamine effect was obtained. The quantities of the agents which produced this extinction effect provided a basis for a comparison of the anti-histamine activity of the compounds assayed.

Figure 1a shows an example of the application of the method to the inhibitory effects produced by acetyl-dehydrophenylalanyl-histamine. Table 1 shows the relative quantities of the different compounds required to extinguish the stimulant effect of 0.02 to 0.05γ of histamine base. We can see that arginine mono-hydrochloride is far less active than other arginine derivatives in which the —COOH or the —NH$_2$ groups or both have been blocked by benzoyl and amide radicals. This might suggest that protecting those groups, the imine =NH group of the guanidyl radical would become entirely free to combine with the chemical cellular receptors for histamine, since there usually is an intramolecular

Fig 1 Isolated Guinea Pig Gut Suspended in a 3 cc Tyrode Bath

At (w)

TABLE I

Relative amounts of different compounds necessary to inhibit the stimulant effect of histamine upon the guinea pig ileum

EXP NO *	COMPOUNDS ASSAYED	AMOUNTS OF THE COMPOUNDS ADDED (cc of M/20)	HISTAMINE ADDED
		γ	
I	Arginine monohydrochloride	0 50	0 02
	Benzoyl l arginine	0 55	0 02
	Benzoyl l arginine amide HCl	0 15	0 02
	Hippuryl nitro l arginine	0 15	0 02
II	Arginine monohydrochloride	0 90	0 03
	Benzoyl l arginine amide HCl	0 20	0 03
	Histidine monohydrochloride	0 20	0 03
	Acetyl dehydrophenylalanyl histamine HCl	0 20	0 03
III	Acetyl dehydrophenylalanyl histamine HCl	0 30	0 01
	Acetyl d l phenylalanyl histamine HCl	0 30	0 01
	Benzoyl l tyrosyl histamine	0 25	0 04
IV	Arginine monohydrochloride	1 50	0 05
	Acetyl dehydrophenylalanyl histamine HCl	0 40	0 05
V	Arginine monohydrochloride	1 0	0 05
	Benzoyl l arginine amide HCl	0 20	0 05
	Acetyl dehydrophenylalanyl histamine HCl	0 25	0 05
	Acetyl d l phenylalanyl histamine HCl	0 20	0 05
VI	Arginine monohydrochloride	0 80	0 03
	Benzoyl l arginine amide	0 15	0 03
	Histidine monohydrochloride	0 15	0 03

* The experiment number denotes a series of assays performed upon the same fragment of intestine, under approximately identical conditions

tendency for free —COOH carboxyl group of arginine to influence the strongly alkaline guanidyl radical (Zervas and Bergmann, 6). To support such interpretation, one might recall the finding of Ackermann and Wasmuth (2) that argininic acid $H_2NH \cdot (:NH) \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH \cdot OH \cdot COOH$ does not inhibit histamine effects. On basis of the above assumption, one might expect that histidine, the histamine compounds assayed, and benzoyl-l, arginine-amide, which have an entirely free =NH imine group, would have a like capacity to inhibit the histamine effects. As can be seen in table 1, there is no substantial difference in the ability of those compounds to counteract histamine.

Fig. 1b, shows the relaxing effect of 0.4 cc. of acetyl-dehydrophenylalanyl-histamine (M/20) upon the sustained contraction produced by 0.1 cc. of histamine solution (1/5 millions). As can be seen, the effect produced by 0.3 cc. of an
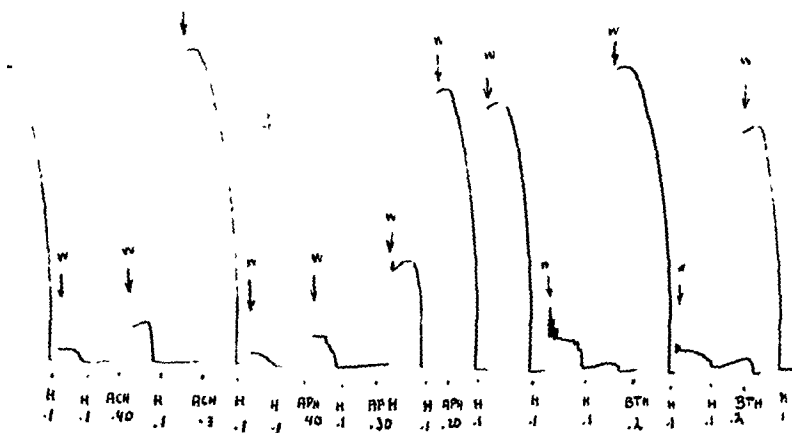
FIG. 2. ISOLATED GUINEA PIG GUT, SAME CONDITIONS AS IN FIG. 1
BTH = Benzoyl-l,tyrosyl-histamine; APH = acetyl-d,l,phenylalanyl-histamine; ACH = acetyl-dehydrophenylalanyl-histamine (acetyl-aminocinnamyl-histamine); H = histamine base (1/5 millions).

acetyl-choline solution (1/10 millions) is only partially prevented by this dose of the histamine compound (ACH).

Fig. 2 shows the inhibitory effect of the histamine compounds upon the effect of 0.05 γ of histamine base. As can be seen, benzoyl-l,tyrosyl-histamine, acetyl-d,l,phenylalanyl-histamine, acetyl-dehydrophenylalanyl-histamine have an almost interchangeable inhibitory effect upon the same dose of histamine. Figs. 3 and 5 show a like effect produced by appropriate amounts of l,arginine monohydrochloride, benzoyl-l,arginine-amide and histidine monohydrochloride. As can be seen, arginine is several times less active than the other compounds studied.

Ackermann and Wasmuth (2) reported that the inhibitory effect of arginine is specific for histamine, since it would not extend over the stimulant action of

choline or acetyl-choline. We have tried the effect of arginine, benzoyl-*l*,arginine-amide and the histamine compounds upon the stimulant effect of a small dose of acetyl-choline. Some of the typical results obtained are shown in fig. 1*b* and fig 4. If it is true that the inhibitory effect upon histamine is more powerful than that upon acetyl-choline, in the case that the dose of arginine is increased over a certain threshold, the acetyl-choline effect is also abolished. This is true,
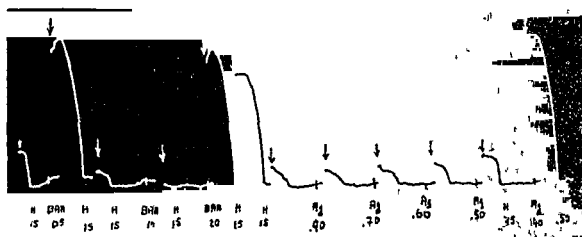
FIG 3 ISOLATED GUINEA PIG GUT

Ag = arginine monohydrochloride (M/20), BAA = benzoyl *l*,arginine-amide (M/20)
H = histamine 1/5 millions
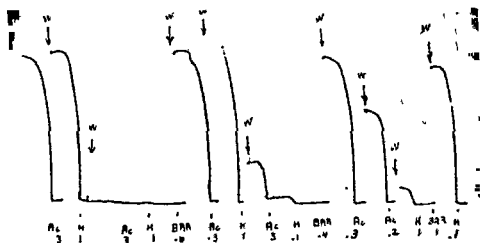
FIG 4 ISOLATED GUINEA PIG GUT

BAA, H and Ac mean M/20 solution of benzoyl *l* arginine amide, 1/5 millions solution of histamine and 1/10 millions solution of acetyl choline respectively

not only as regards the arginine effect, but also the effects of benzoyl-*l*,arginine-amide, acetyl-dehydrophenylalanyl-histamine, acetyl *d*,*l*,phenylalanyl-histamine and histidine monohydrochloride

At present, there is no satisfactory explanation for the inhibitory effect of those compounds upon the acetyl-choline stimulant effect. Notwithstanding that, we can recall that atropine, the specific inhibitor for acetyl-choline, inhibits in a

certain extent also the histamine effects (Feldberg, 7; Riesser, 8; Code, 9).   This
shows that there is a possible interdependence between the sites of attack of
both pharmacological agents.

Effect upon the circulatory apparatus of the cat.   Ackermann (4) reported that
injecting a proper amount of histamine in a mixture with arginine (0.6 gm. of
arginine per kilo weight, in dogs), would fully inhibit the vaso-depressor action
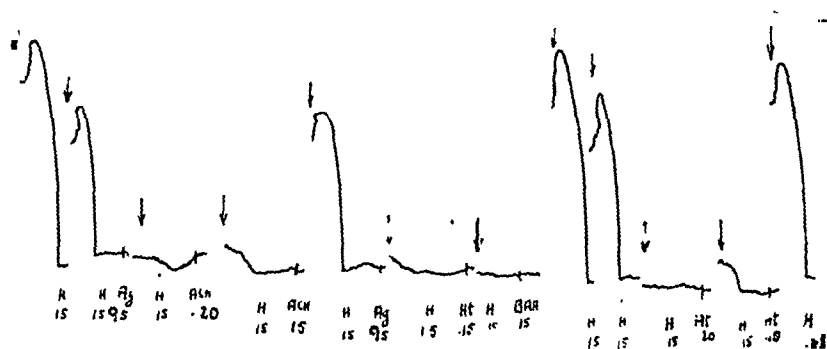
FIG. 5. ISOLATED GUINEA PIG GUT

Ag and BAA, arginine monohydrochloride and benzoyl-*l*,arginine-amide (M/20) respec-
tively.   Ht = histidine monohydrochloride M/20.
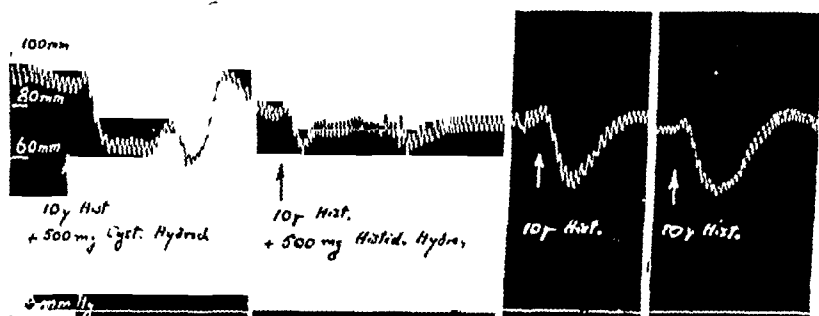
FIG. 6. CAROTID BLOOD PRESSURE IN THE CAT (2.9 KGM. BODY WEIGHT)

At the arrows, 10 γ of histamine were injected in mixture with cysteine hydrochloride,
histidine monohydrochloride and saline.   There was no protection afforded by cysteine and
a partial protection by histidine.

of the drug.   More recently, Edholm (10) showed that a long treatment with
histidine would diminish the response of the circulatory apparatus of the cat to
an ulterior injection of histamine.

In view of the possibility that the histamine effect upon the circulatory ap-
paratus of mammals might be explained by its effect either upon the smooth
musculature of the hepatic veins (in the dog), or upon the smooth muscle coats
of the pulmonary artery (in the cat), it has been found advisable to try the in-

hibitory effect of arginine and histidine upon the intravenously injected histamine. The results obtained, some of which are presented in figures 6, 7, and 8 were rather deceiving, since even against very small amounts of histamine
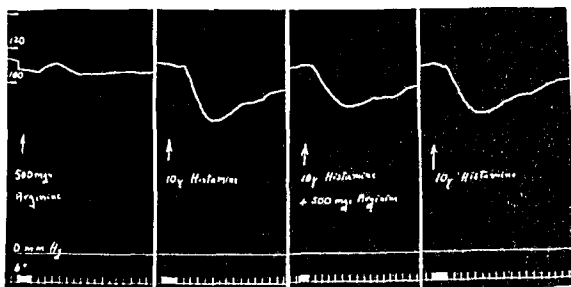
FIG. 7 CAROTID BLOOD PRESSURE IN THE CAT (2 95 KGM. BODY WEIGHT)
Effects produced by arginine alone, histamine alone (10 γ) and histamine (10 γ) + arginine monohydrochloride (500 mgm)
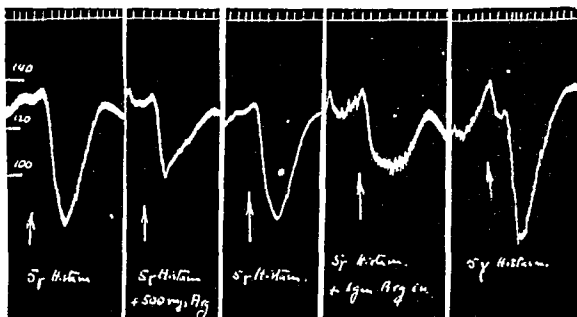
FIG 8 CAROTID BLOOD PRESSURE IN THE CAT (3 8 KGM BODY WEIGHT)
Effects produced by intravenous injection of histamine (5 γ), histamine (5 γ) + arginine monohydrochloride (500 mgm) and histamine (5 γ) + arginine monohydrochloride (1 0 gm)

(2.5 to 10γ), the protection afforded by 500 mgm. of arginine or histidine was slight or non-existent. Even when greater amounts of the amino acids (0.5 to 1 0 gm) were injected, the counteracting effects were still slight

As shown in table 1, to counteract 0.02 to 0.05 γ of histamine, one has to add 0.5 to 1.0 cc. of M/20 solution of argine monohydrochloride to the perfusing bath containing the guinea pig ileum. Calculated in mgm. per γ of histamine, one would have to inject 0.5 to 1 gm. of arginine monohydrochloride to fully counteract the effects of 2 to 4 γ of histamine injected intravenously. The experiments have shown that 500 mgm. to 1.5 gm. of arginine monohydrochloride, only partially inhibited the effects of 2.5 to 5 γ of histamine injected in a mixture with the amino acid. Only in one occasion it has been possible to counteract the effect of 5 γ of histamine with 500 mgm. of histidine monohydrochloride (fig. 6). In every other case, the injection of 1.0 to 1.5 gm. of histidine was even followed by a definite aggravation of the histamine effects. This was shown to be due to the hypotensor effect of histidine itself, when injected intravenously in doses above 1.0 gm. The possibility that minute amounts of histamine would impurify the histidine crystals used has not been ruled out by further experiments, due to the difficulty to separate histamine from histidine. We have tried some of Hanke and Koessler's procedures (11), without very much success.

DISCUSSION. The experiments referred to above have shown a new possibility to increase the number of anti-histamine substances. If one blocks the pharmacological activity of histamine by combining its NH_2 group with the COOH group of an amino-acid, the imine group ($=NH$) of the imidazol ring being left intact, the resulting compound would display an anti-histamine effect. This was definitely shown by assaying the compounds: acetyl-dehydrophenylalanyl-histamine, benzoyl-l,tyrosyl-histamine and acetyl-d,l,phenylalanyl-histamine, all of them displaying a definite anti-histamine effect when tried upon the isolated guinea pig gut.

Under a pharmacological point of view it was interesting to test further on the theory formulated by Ackermann (4) that the inhibitory effect of arginine, histidine and cysteine upon the histamine effects, was due to the fact that those compounds had an imine ($=NH$) or sulfhydryl ($—SH$) group which might compete with the like imine group of histamine, on its anchoring capacity upon the receptors of the cell effectuors. The results of the experiments presented in this paper conform satisfactorily with Ackermann's view. Under this respect it is worthy of note that benzoyl-l,arginine-amide, benzoyl-l,arginine and hippuryl-nitro,l,arginine are definitely more potent than arginine itself. If one recalls that Ackermann and Wasmuth (2) found argininic acid almost inactive, the following series of decreasing inhibitory potency, might hold for arginine derivatives:

benzoyl-l,arginine-amide $>$ benzoyl-l,arginine $>$ arginine monohydrochloride $>$ argininic acid.

This shows that blocking either the $—NH_2$ group or the $—COOH$ group or both, definitely increases till a certain maximum the inhibitory effect of the arginine compounds upon the histamine effects on the guinea pig gut.

The above experiments have shown that approximately 0.02 to 0.05 γ of histamine base, can be *in vitro* counteracted by 5 to 10 mgm. of arginine. This

ratio, 250 mgm of the amino acid to 1 γ of histamine base, might explain the failure of arginine to protect the guinea pig against the lethal effects of histamine, as has been consistently shown in several experiments performed by ourselves Since the lethal dose of histamine for guinea pigs amounts to 200 to 500 γ of histamine injected intravenously, it would be necessary to inject simultaneously, so large amounts of arginine (50 to 100 grams!) that the experiment could not obviously be done

This shows the limitations for the practical use of arginine as an anti histamine therapeutic agent Since the proofs are being accumulated that liberated hista mine is the causative factor in human allergy (Rose, 12, Rose, 13, Katz and Cohen, 14, Katz, 15) and as Ackermann and Wasmuth (3) have shown the inhibitory effect of arginine upon *in vitro* anaphylactic reaction, one might be induced to try arginine as a therapeutic agent against human allergy   Conclud ing from the animal experiments presented above, it seems rather unlikely that arginine might have any significance for the treatment of allergic diseases, since one would have to inject intravenously huge amounts of it to counterbalance the effects of even small amounts of histamine liberated from the tissues   There is, however, two possibilities that might encourage one for using arginine as a therapeutic agent in human allergy 1) that the liberated histamine, effective in much smaller doses than injected histamine, might be counterbalanced by fewer grams of arginine, 2) any possible unexpected effect upon chronic administration of the amino acid

As regards the first item, it is interesting to recall the findings of Schild (16) who has shown that liberated histamine is about 15 times as effective as added histamine, upon the isolated guinea pig gut Consequently there will be the possibility to reduce 15 times the necessary amount of arginine to inhibit allergic responses, as compared to similar responses to histamine   The other possibility is the one investigated by Edholm (10) who has shown that a long treatment of cats with histidine might contribute to reduce the sensitivity of this animal species to the histamine effects   The difficulty to have histidine preparations absolutely free of histamine traces, however, might suggest that the increased resistance obtained by Edholm against histamine, after histidine injections, might be due to an increase of resistance produced by chronic administration of minute amounts of histamine, as obtained in guinea pigs by Farmer (17), Karady (18), Essex and Horton (19) and others   Further experiments will throw light on this important point

SUMMARY

A method of studying quantitatively the anti histamine effects of histamine, arginine and histidine derivatives upon the isolated guinea pig gut is devised Among the arginine derivatives studied—benzoyl *l*,arginine amide, benzoyl *l*, arginine, hippuryl nitro,*l*,arginine and arginine monohydrochloride—the ben zoyl *l*,arginine amide was many times more active than arginine monohydro chloride   Since histidine monohydrochloride displayed a similar inhibitory potency, there was assumed that the anti histamine effect which probably de

pends on the =NH (imine) group of those amino acid derivatives, attains a maximum corresponding to the anti-histamine effect of benzoyl-*l*,arginine-amide.

The histamine compounds studied—acetyl-dehydrophenylalanyl-histamine, benzoyl-*l*,tyrosyl-histamine and acetyl-*d*,*l*,phenylalanyl-histamine—are pharmacologically inactive, while still retaining its imine (=NH) group entirely free. They have shown an anti-histamine potency of the order of magnitude of histidine monohydrochloride and benzoyl-*l*,arginine-amide. The perfect agreement of those results with Ackermann's view that the inhibitory effect of arginine and histidine depends on a competition between the imine group of those amino acids with the like imine group of histamine on its anchoring capacity upon the cellular receptors, is emphasized.

On basis of those quantitative results obtained upon the isolated guinea pig ileum, it was inferred that 0.5 to 1.5 gm. of either arginine or histidine would be enough to counteract the depressor effect of 2 to 5 γ of histamine base. The experiments performed in the cat gave rather deceiving results. Even when a sufficient excess of arginine or histidine were injected in mixture with small doses of histamine the counteracting effect was small or non-existent. Some times the injection of 1.5 gm. of arginine or histidine in mixture with histamine, produced an aggravation of the effects of the latter.

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